Universidade de Aveiro Departamento de Biologia
Ano 2015

Mariana Lourenço Mocho Fernandes Gomes

LIFESTYLE IMPACT ON HUMAN SPERM FUNCTION

IMPACTO DO ESTILO DE VIDA NA FUNÇÃO DOS ESPERMATOZOIDES HUMANOS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Margarida Sâncio da Cruz Fardilha, Professora auxiliar convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro

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palavras-chave

Stress oxidativo, estilo de vida, espermatozoides, espermograma, função das glândulas acessórias, oxidação proteica, enzimas antioxidantes

resumo

O stress oxidativo (OS) tem sido considerado uma causa importante da infertilidade masculina, que está envolvida em cerca de metade dos casos de infertilidade. O OS induzido pelas espécies reativas (RS) é prejudicial para os espermatozoides, levando a lesões em várias biomoléculas, como os lípidos, proteínas e DNA. Alterações no estilo de vida, como o consumo excessivo de álcool e tabaco, induzem o OS e têm sido extensivamente estudadas devido aos seus efeitos negativos ao nível do sistema reprodutor masculino.

O objetivo deste estudo foi analisar o impacto de alterações agudas no estilo de vida, nomeadamente o consumo de álcool e tabaco, na qualidade seminal, na função das glândulas acessórias e no equilibrio oxidativo dos espermatozoides. Para além disso, outro objetivo deste trabalho foi avaliar a possível relação entre os parâmetros de OS e os parâmetros seminais analisados.

Estudantes masculinos, em idade fértil, que participaram nas festividades académicas, doaram uma amostra de sémen em três períodos de tempo: antes e uma semana e três meses após as festividades académicas. A análise básica ao sémen foi realizada e, posteriormente, as amostras foram processadas. A função das glândulas acessórias foi avaliada, assim como determinada a capacidade antioxidante total das células, a expressão das enzimas antioxidantes superóxido dismutase 1 e glutationa peroxidase 4 e a presença de grupos carbonilo e 3-nitrotirosina.

Os resultados indicam que uma diminuição na qualidade seminal, demostrada por um decréscimo na motilidade progressiva dos espermatozoides e na concentração de α-glucosidase neutra e um aumento nos defeitos da cauda, ocorre devido a alterações no estilo de vida. A capacidade antioxidante total das células e as variações ao nível da oxidação proteica demonstram também ser dependentes do consumo de alcool e tabaco. Foram também verificadas algumas correlações entre os parâmetros analisados que poderão ser importantes numa perspetiva clínica.

Concluindo, alterações no estilo de vida são responsáveis pela diminuição da qualidade seminal e pelo aumento de modificações proteicas, o que pode levar consequentemente a um decréscimo do potencial de fertilização.

keywords

Oxidative stress, lifestyle; spermatozoa, basic semen analysis, accessory glands function; protein oxidation; antioxidant enzymes

abstract

Oxidative stress (OS) is believed to be an important cause of male infertility, which accounts for about half of all infertility cases. Reactive species (RS)-induced OS is detrimental to spermatozoa, leading to the damage of many biomolecules, such as lipids, proteins and DNA. Several lifestyle factors, such as alcohol and tobacco consumption, are known to induce OS and have been studied for their negative effects on male reproductive system.

The aim of this study was to evaluate the impact of acute lifestyle changes, namely alcohol and tobacco consumption, on semen quality, accessory glands function and oxidative balance of sperm cells. Furthermore, the correlation between the OS parameters analyzed and the basic semen parameters was also assessed.

Male students, in reproductive age, who participated in the academic festivities, donated a semen sample at three time points: before and one week and three months after the academic festivities. Basic semen analysis was performed and, subsequently, semen samples were processed. Acessory glands function was evaluated and OS was analyzed through measurement of the total antioxidant capacity of the sperm cells and through determination of the expression of antioxidant enzymes glutathione peroxidase 4 and superoxide dismutase 1. The impact of ROS in spermatozoa was also assessed through the determination of the protein carbonyl and 3-nitrotyrosine groups.

The results indicate that a decrease in semen quality, demonstrated by a decrease in progressive motility and neutral α-glucosidase concentration and an increase in tail defects, occurs due to lifestyle alterations. The total antioxidant status of sperm cells and variations in protein oxidation levels are dependent on the alcohol and tobacco consumption. Moreover, some correlations were observed between the studied parameters, which may be useful in a clinical perspective.

In conclusion, the lifestyle alterations are responsible for a decrease in semen quality and by an increase in protein modifications, which may consequently lead to a decrease in fertilizing potential.

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List of abreviations

17β-HSD	17beta-hydroxysteroid dehydrogenase
3-NT	3-nitrotyrosine
3β-HSD	3beta-hydroxysteroid dehydrogenase
4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxydeoxyguanosine
ABP	androgen binding protein
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AOT	acridine orange test
APS	ammonium persulfate
ART	assisted reproductive techniques
ATP	adenosine Tri Phosphate
BaP	benzo-(a)pyrene
BCA	bicinchoninic acid
BSA	bovine serum albumin
BTB	blood-testis barrier
CAT	catalase
cGMP	cyclic guanosine monophosphate
CK	creatine kinase
COMET	single-cell gel electrophoresis
DAPI	4',6-diamidino-2-phenylindole
DHA	docosahexaenoic acid
DNPH	2,4-Dinitrophenylhydrazine
ETC	electron transport chain
FSH	follicle stimulating hormone
G6PDH	glucose-6-phosphate dehydrogenase
GnRH	gonadotrophin-releasing hormone
GPx	glutathione peroxidase
GR	gluthatione reductase
GSH	reduced gluthatione

GSSG	oxidized glutathione
GST	gluthatione-S-transferase
HPG	hypothalamic-pituitary-gonadal
IL	interleukin
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
NADPH	nicotinamide adenine dinucleotide phosphate
NAG	neutral α-glucosidase
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
NOX	NADPH oxidase
OGG1	8-oxoguanine glycosylase 1
OS	oxidative Stress
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffer saline
PKA	protein Kinase A
PLA_2	phospholipase A_2
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
RS	reactive Species
RT	room temperature
SCSA	sperm chromatin structure assay
SDS	sodium dodecyl sulfate
sGC	soluble guanylyl cyclase
SHBG	sex hormone-binding globulin
SOD	superoxide dismutase
StAR	steroidogenic acute regulatory protein
TAS	total antioxidant status
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
TBS	tris Buffered Saline
TBS-T	tris Buffered Saline and Tween-20
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
TP	time point
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
Tyr	tyrosine
ZIP	zinc-regulated *transporters*, Iron-regulated *transporter*-like Proteins

1. Introduction

1.1. Human sperm

1.1.1. Spermatogenesis and the journey to epididymis

Spermatogenesis is a biological process that occurs within seminiferous tubules in the testis and leads to the transformation of A-spermatogonium (stem cell) into spermatozoa. It initiates at puberty but a preparatory period begins before birth, with male sexual development, and remains until adulthood (O'Donnell et al. 2001; Hess & De Franca 2008). The production of sperm cells is regulated by extrinsic stimuli provided by hypothalamus and pituitary gland, which release several hormones, including follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Weinbauer et al. 2010). Spermatogenesis is also controlled by testosterone, neurotransmitters and growth factors, which are involved in the maintenance of Sertoli cells (Rato et al. 2012), in the transport of spermatozoa and in the regulation of blood flow (Holstein et al. 2003).

The human testis is formed by several conical tubules consisting of seminiferous tubules and interstitial tissue (O'Donnell et al. 2001). Seminiferous tubules consist of several germinal cell types and Sertoli cells, the unique somatic cell type (Holstein et al. 2003; Sofikitis et al. 2008). Adjacent Sertoli cells form tight junctions, which seal the cells to each other, forming the blood-testis barrier (BTB). Thus, two regions are formed: the basal compartment, where early germ cells are located, and the adluminal region composed by late maturing germ cells (Weinbauer et al. 2010). This barrier does not allow the passage of many molecules and substances (hormones, environmental toxicants, drugs, chemicals and others), creating a protective and ideal environment for the development of germ cells (Cheng & Mruk 2012). Structural and nutritional support of germ cells, intervention in spermiation, production of essential substances for the regulation of spermatogenesis, secretion of androgen binding protein (ABP) and interaction with Leydig cells are other functions attributed to Sertoli cells (Holstein et al. 2003; Alves et al. 2013). Leydig cells are found in the interstitial tissue, together with immune cells, blood and lymph vessels, nerves, fibroblasts and connective tissue (Weinbauer et al. 2010) and their main function is testosterone production, which is necessary for the development of secondary sex characteristics in puberty and the onset of spermatogenesis (Svechnikov et al. 2010).

Spermatogenesis and the production of male steroid hormones occur in seminiferous tubules and in the interstitial compartment respectively, which are two compartments

morphologically and functionally distinct. However, these compartments are both regulated by hypothalamus-pituitary gland axis (Weinbauer et al. 2010). The hormonal regulation of the testicular function is represented in **Figure 1**. Gonadotrophin-releasing hormone (GnRH) is secreted by hypothalamus, which stimulates the release of FSH and LH from pituitary gland (McLachlan 2000; Shalet 2009). LH acts in Leydig cells, stimulating the release of testosterone. In turn, testosterone acts on androgen receptor (AR), which only exists in Leydig, Sertoli and peritubular cells (O'Donnell et al. 2001; Walker 2009). On the other hand, FSH and LH-stimulated testosterone act on Sertoli cells to regulate spermatogenesis by stimulating the production of many factors important to the development of germ cells (O'Donnell et al. 2001; Hess & De Franca 2008). Inhibin B is mainly produced by Sertoli cells and seems to negatively regulate (negative feedback) the synthesis and secretion of FSH (Kumanov et al. 2006).

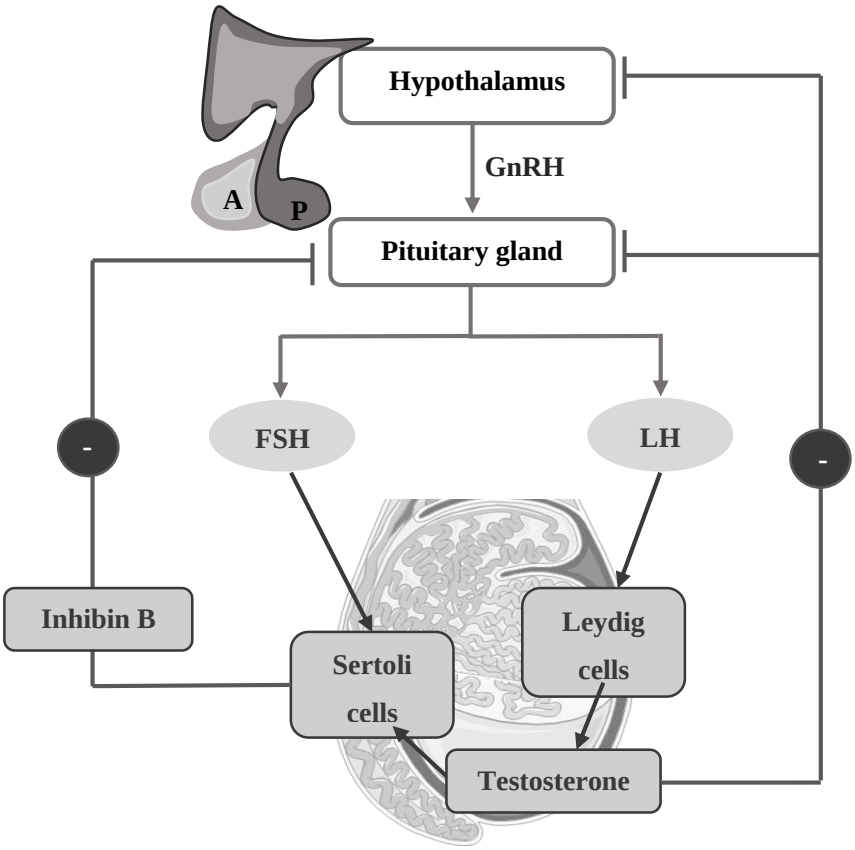

Figure 1 - Hormonal regulation of spermatogenesis. GnRH is secreted by hypothalamus, which stimulates the release of FSH and LH from pituitary gland. LH acts in Leydig cells, stimulating the release of testosterone, which, in turn, will act on AR. FSH and LH-stimulated testosterone act on Sertoli cells to regulate spermatogenesis by stimulating the production of many factors important to the development of germ cells. Inhibin B is mainly produced by Sertoli cells and seems to negatively regulate (negative feedback) the synthesis and secretion of FSH. Figures were produced using Servier

Medical Art. **GnRH:** Gonadotropin-releasing hormone; **FSH:** Follicle-stimulating hormone; **LH:** Luteinizing hormone; AR: androgen receptor; **A:** Anterior pituitary; **P:** posterior pituitary

Spermatogenesis leads to a daily production of more than 200 million of spermatozoa in human testis. Six different stages of spermatogenesis (**Figure 2**) are distinguished in human seminiferous epithelium (Sutovsky & Manandhar 2006). In human germinal epithelium, many types of spermatogonia are present close to the basal lamina: A pale-, A dark- and B-spermatogonia (Clermont 1966; Holstein et al. 2003). A dark-spermatogonia are stem cells that can produce A pale-spermatogonia under FSH and testosterone stimulation (Nieschlag & Behre 2004). A pale-spermatogonia multiplicate in successive mitoses to produce B-spermatogonia (Holstein et al. 2003; Amann 2008). B-spermatogonia enter mitosis forming two primary (preleptotene) spermatocytes, the largest cells of the seminiferous epithelium (Holstein et al. 2003; Hess & De Franca 2008). Leptotene spermatocytes are formed, after preleptotene spermatocytes enter meiosis, and pass through the blood-testis barrier, reaching the adluminal compartment. The progression of prophase in this compartment results in zygotene and pachytene primary spermatocytes, which suffer a duplication in DNA content, condensation and paring of homologous chromosomes and crossing over (Holstein et al. 2003; Sutovsky & Manandhar 2006). Spermatocytes are not independent cells, since they are connected to each other by cytoplasmic bridges (Grootegoed et al. 2000). Then, secondary spermatocytes are formed and enter meiosis II to form haploid cells designated round spermatids. These cells suffer many cytological events that lead to the elongation and condensation of sperm nucleus. The condensation of nucleus is possible due to the removal of nucleossomal histones and their replacement by protamines (Grootegoed et al. 2000). Covering the sperm nucleus is the acrosome, which is formed from Golgi apparatus and depends upon its capacity to produce the enzymatic machinery that will be present in this structure (Hess & De Franca 2008). One of the centrioles of the round spermatid forms the microtubules that will become the axoneme and consequently the flagellum (de Kretser et al. 1998). Only one half of mitochondria present in spermatids obtain a reinforced outer membrane, named mitochondrial capsule, and forms a helical mitochondrial sheath (Sutovsky & Manandhar 2006). Thus, the round spermatids differentiate into mature or elongated spermatids in a process called spermiogenesis. This process is concluded when spermatozoa are released to the lumen of the seminiferous tubule (spermiation). Excess of cytoplasm of spermatids is eliminated by Sertoli cells (Hess & De Franca 2008).

After being release to the seminiferous lumen, spermatozoa are conducted through the rete testis to the epididymis. The transport to the epididymis is assisted by the pressure exerted from sperm mass production in the testis, fluid secretion from Sertoli cells, contractive movements from testicular capsule and myoid layer and cilia movements present in epithelium of the efferent ducts (Sutovsky & Manandhar 2006). Epididymis is composed of three different regions (caput, corpus and cauda), each one with a diverse function. Early and late maturational events occur in caput and corpus region, respectively, and cauda region functions as a local to storage mature spermatozoa (Cornwall & Horsten 2007). During migration from caput to cauda region, spermatozoa suffer biochemical, morphological and physiological alterations to become motile and mature (fertile) spermatozoa. Changes in spermatozoa include modifications in plasmatic membrane, acquirement of proteins released by epididymal epithelium and phosphorylation of specific proteins important for maturational steps (Toshimori 2003; Cornwall & Horsten 2007; Cornwall 2009).

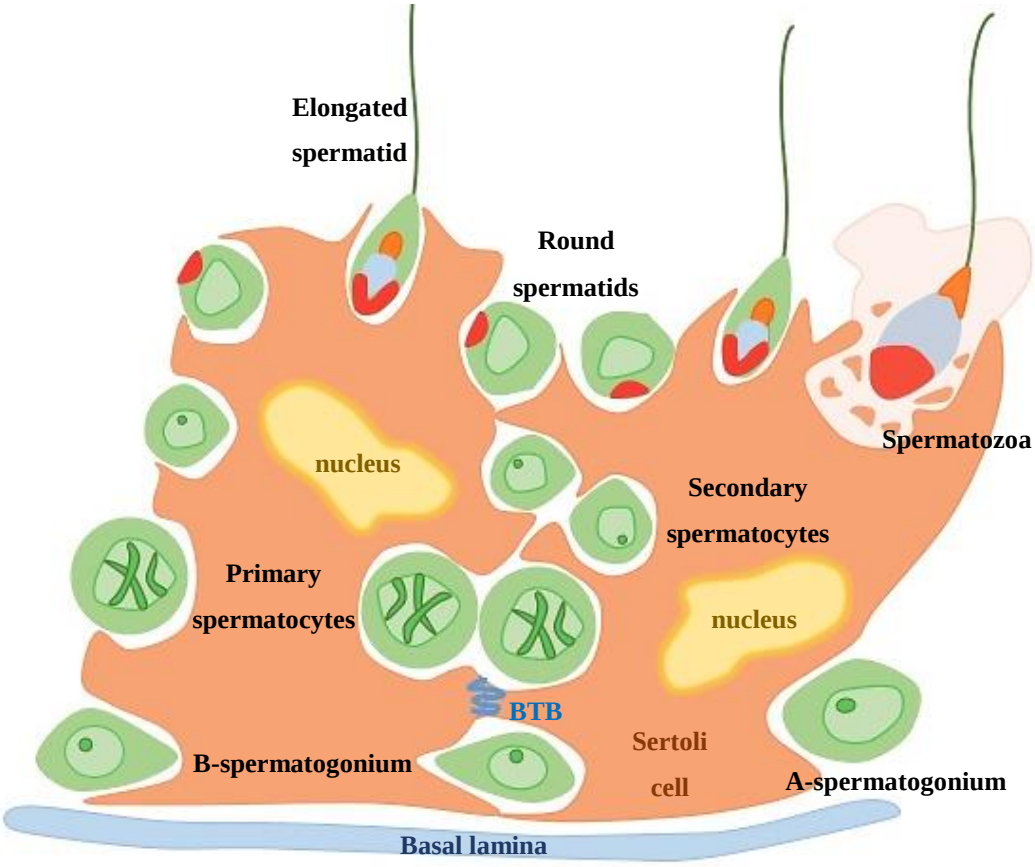

Figure 2 - Schematic illustration of spermatogenesis. A schematic cross-section of a testicular tubule, showing the germ cells (green) at different stages of maturation embedded in Sertoli cells (orange). The tight junctions between Sertoli cells form the BTB and define two compartments: the

basal compartment, where early germ cells are located, and the adluminal region composed by late maturing germ cells. **BTB:** blood-testis barrier.

When spermatozoa are ejaculated, they are suspended in a fluid medium known as seminal plasma (SP), which provides nutritional and protection support to spermatozoa (du Plessis et al. 2013). SP is constituted by the fluids of the cauda epididymis and accessory sexual glands, such as prostate and seminal vesicles (Rodríguez-Martínez et al. 2011). Human SP is examined for biomarkers to assess gland function, such as fructose, zinc and α-glucosidase (WHO 2010; Rodríguez-Martínez et al. 2011). Seminal vesicles fluid is enriched in fructose (WHO 2010), providing to spermatozoa the required substrate for energy production (Andrade-Rocha 2003; du Plessis et al. 2013). Prostate releases zinc, important for chromatin stability (Rodríguez-Martínez et al. 2011), citric acid and acid phosphatase (WHO 2010), while epididymis contributes almost exclusively with α-glucosidase, important for sperm metabolism (Andrade-Rocha 2003). Two isoforms of α-glucosidase are found in SP: a neutral form, produced exclusively by epididymis, and an acidic form, originated from the prostate (WHO 2010).

1.1.2. Human spermatozoa

The human spermatozoon (**Figure 3**) is a differentiated and motile cell with a small amount of cytoplasm and organelles. It is mainly composed of an oval sperm head, a midpiece and a tail, also designated by flagellum (Pedersen 1969; Ramalho-Santos 2012).

The sperm nucleus is found in the head, where histones are replaced by protamines in spermiogenesis to allow a well compaction of the nucleus and consequently a reduction on sperm volume (Roux et al. 1988). This morphological change allows better aerodynamic properties, facilitating the fertilization of the oocyte. The perinuclear theca, the major cytoskeletal component of sperm head, surrounds and protects the nucleus and contains many proteins that are responsible for the shaping of sperm head and egg activation (Oko & Sutovsky 2009; Toshimori 2009). Acrosome is found over the nucleus and contains many digestive enzymes, such as acrosin, that plays an important role in sperm penetration through *zona pellucida* (Green & Purves 1984; Ramalho-Santos et al. 2007). Before sperm undergo acrosome reaction in the female reproductive tract, spermatozoa suffer additional maturational steps called capacitation

and hyperactivation. These events are sequential (Toshimori 2009) and both lead to acrossomal exocytosis near the oocyte and the passage and fusion with the oolema (Sutovsky 2010). Equatorial segment is a complex located in the final region of the acrosome and is involved in the fusion of sperm membrane with oolema during fertilization (Toshimori 2009).

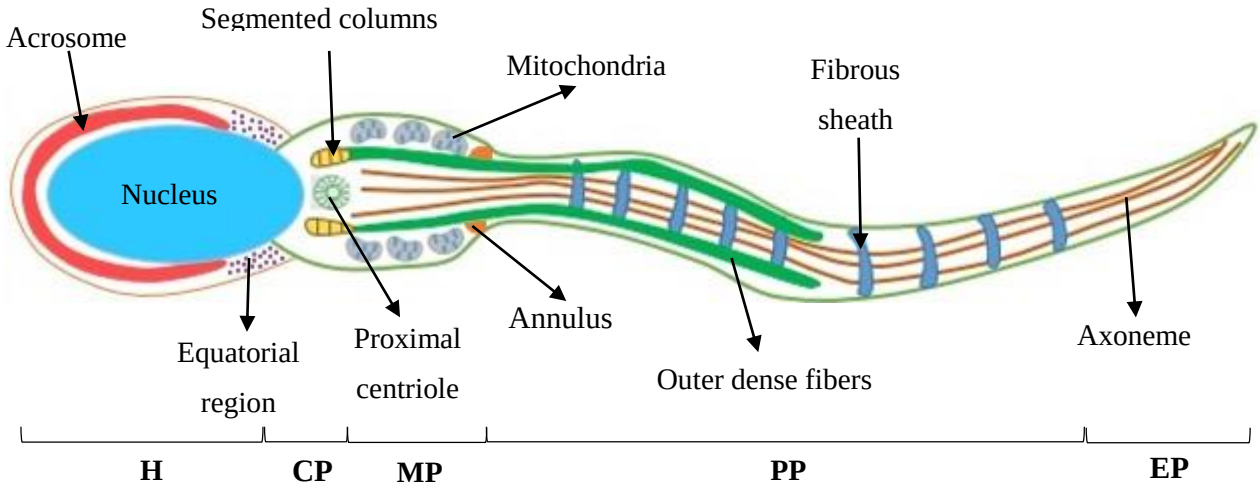

Figure 3 - Schematic representation of human spermatozoa. The human spermatozoon is a differentiated and motile cell, composed of an oval sperm head, a midpiece and a tail. The sperm nucleus is found in the head, where histones were replaced by protamines in spermiogenesis. Acrosome is found over the nucleus and contains many digestive enzymes, such as acrosin. Sperm tail is divided into four main segments: connecting piece, midpiece, principal piece and end piece. The proximal centriole and segmented columns in the connecting piece originate the axoneme and the outer dense fibers respectively. The midpiece is composed by a helix of mitochondria, important for ATP production. **H:** Head; **CP:** Connecting piece; **MP**: Midpiece; **PP:** Principal piece; **EP**: End piece.

Sperm tail is divided into four main segments: connecting piece, midpiece, principal piece and end piece (Hermo et al. 2010). The connecting piece is composed of nine segmented columns that enclose the proximal centriole. Segmented columns and proximal centriole originate nine outer dense fibers and the axoneme, respectively, both present in the other flagellum segments (Chemes 2012). Axoneme is a cytoskeletal structure composed of 9 outer doublet microtubules and 2 central singlet microtubules (Inaba 2011). Midpiece is the connection between the sperm head and tail and is composed by a helix of 75-100 mitochondria, creating a structure designated by mitochondrial sheath (Sutovsky & Manandhar 2006). In this

tail segment, outer dense fibers are also present. ATP production through oxidative phosphorylation in mitochondria occurs in the midpiece, however this molecule might not be able to travel along the entire tail through diffusion. Recent studies demonstrated that glycolysis, which occur along the sperm tail, is the primary source for sperm motility (Nascimento et al. 2008) and spermatozoa can also obtain energy through fatty acid oxidation (Amaral, Castillo, et al. 2013). Mitochondrial ATP seems to be used in specific situations, such as sperm capacitation (Ramalho-Santos et al. 2007; du Plessis et al. 2015). The annulus, a ring of dense material, is found distally to mitochondrial sheath and is a physical barrier between midpiece and principal piece (Hermo et al. 2010). In the principal piece, the axoneme is surrounded by outer dense fibers and also by the fibrous sheath (Inaba 2011), composed by two longitudinal columns connected by a series of transverse ribs (Sutovsky & Manandhar 2006). Finally, the end piece contains the microtubules of axoneme and the end of the fibrous sheath (Sutovsky & Manandhar 2006).

1.2. Oxidative stress and human sperm

Male infertility is a clinical problem which has been attributed in part to oxidative stress (OS) (Benedetti et al. 2012). The male factor is exclusively responsible for infertility in 20% of the cases, but it also contributes to another 30-40% of cases together with the female factors (Hamada et al. 2011). In Portugal, assisted reproductive techniques (ART) have increased along the years and infertility seems to affect 13 to 18% of couples in reproductive age, which represents about 290 000 couples (Silva & Calhaz-Jorge 2010).

Reactive species (RS) are known to induce OS in many forms of male infertility, inciting chemical and structural alterations to sperm proteins, lipids and DNA (Gharagozloo & Aitken 2011). OS is defined as an imbalance between the production of RS and the capacity of antioxidant defense system to neutralize these compounds (Agarwal, Virk, et al. 2014). RS generation in spermatozoa occurs mainly in mitochondria due to the reduction and oxidation reactions during the generation of proton gradient across the inner mitochondrial membrane. The two main sources are the auto-oxidation of intermediate semiquinones in complexes I or III, which are non-enzymatically oxidized by molecular O_2 to $O_2^{\cdot -}$ (Koppers 2012). RS include an extensive group of molecules that include radicals, non-radicals (neutral) or ionic, as described in **Table 1** (Agarwal et al. 2008).

Table 1 – Endogenous reactive species produced in cells.

Endogeneous Species	Reactive Oxygen Species	Reactive Nitrogen Species
Radical	$O_2^{\cdot -}$; $OH^{\cdot}$; $LOO^{\cdot}$	$NO^{\cdot}$; $NO_2^{\cdot}$
Ionic	$ONOO^-$	NO- ; $NO^{\cdot}$
Neutral	H_2O_2 ; O_3 ; $^1O_2$; LOOH	N_2O_3 ; N_2O_4

Semen contains different types of cells that can produce RS: mature and immature spermatozoa, cells at different stages of spermatogenesis, leukocytes, Sertoli and epithelial cells. Leukocytes and defective spermatozoa are suggested as the main sources of RS in seminal plasma (Chen et al. 2013; Agarwal, Virk, et al. 2014). Leukocytes, under normal conditions, produce about 1000 times more RS than sperm cells, which is an important defense mechanism against inflammation and infections (Walczak-Jedrzejowska et al. 2013). The white blood cells responsible for the major production of RS in seminal plasma are peroxidase-positive leukocytes, which include polymorpho-nuclear leukocytes and macrophages (Agarwal, Virk, et al. 2014). These cells release pro-inflammatory cytokines, such as interleukin (IL)-6, IL-8 and tumor necrosis factor α (TNF-α), and possibly nuclear factor kappa B (NF-κB), which induce OS (Tremellen 2008; Chen et al. 2013). Immature spermatozoa exhibit excess cytoplasmic droplet in the midpiece region, due to an arrest in spermiogenesis and incomplete cytoplasmic extrusion (Rengan et al. 2012). These cytoplasmic droplet are enriched with many cytoplasmic enzymes, such glucose-6-phosphate dehydrogenase (G6PDH) and creatine kinase (CK). The first enzyme, through the hexose monophosphate shunt, leads to a larger production of NADPH, which is used as a substrate for the generation of RS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases within sperm membrane (Tremellen 2008; Chen et al. 2013).

Some pathologies are also responsible for the increase of RS which affect the sperm cells. Varicocele causes an irregular dilatation, elongation and tortuosity of the veins of spermatic cord, which leads to an increase in OS and consequently to male infertility (Agarwal et al. 2012). Many studies have demonstrated that men with varicocele produce excessive amounts of nitric oxide within spermatid cord, which is responsible for spermatozoa dysfunctions, DNA damage and lipid peroxidation (reviewed in (Cocuzza et al. 2007)). Many lifestyle factors, such as smoking and alcohol consumption, and environmental factors, may also contribute to RS production, which will be discussed in more detail in the next section.

Mature spermatozoa also produce RS via mitochondria, namely superoxide anion ($O_2^{\cdot-}$) in the electron transport chain (ETC) (Amaral, Lourenço, et al. 2013). Furthermore, the NADPH oxidase system, localized in sperm membrane, is another source of RS in sperm cells (Walczak-Jedrzejowska et al. 2013). A calcium-dependent NADPH oxidase named NOX5, found in midpiece, acrossomal and tail regions of spermatozoa, also generates RS, namely $O_2^{\cdot-}$, and is thought to be the main enzyme responsible by RS production (Amaral, Lourenço, et al. 2013; Chen et al. 2013).

Although high amounts of RS damage spermatozoa, it is known that low concentrations are essential to normal physiological processes (capacitation, acrosome reaction, sperm-oocyte fusion as well as sperm motility, morphology and viability) (Doshi et al. 2012). Sperm capacitation is an irreversible process that leads to the remodeling of sperm plasma membrane, facilitating the acrosomal exocytosis and the fusion with oocyte (Sutovsky 2010). In this process a cascade of phosphorylating events occurs, which activate protein kinase A (PKA) leading to the phosphorylation of Tyr-residues (Donà et al. 2010). A recent study examined the relationship between the phosphorylation of Tyr residues and endogenous RS generation during sperm capacitation. The results showed the occurrence of a 2-peak pattern, which was chronically related with the phosphorylation of Tyr residues in sperm head and tail and the acrosome reaction (Donà et al. 2010). Together with capacitation, spermatozoa must suffer hyperactivation and acrosome reaction to achieve proper fertilization. When spermatozoa become hyperactivated, due to stimulation by $O_2^{\cdot-}$, flagellar beat becomes asymmetrical and with higher amplitude in order to spermatozoa detach from the oviductal epithelium, reach the site of fertilization and penetrate the cumulus and *zona pellucida* (Turner 2003). Subsequently, spermatozoa undergo acrosome reaction at the time of fertilization in order to create a path through the *zona pellucida* of the oocyte (Hermo et al. 2010), in a process where it seems that H_2O_2 plays an important role (Aitken & Fisher 1994). Low amounts of RS are involved in acrosome reaction through phosphorylation of sperm plasma membrane proteins (Agarwal, Virk, et al. 2014) and also by activating phospholipase A2 (PLA_2), which increases the membrane fluidity (reviewed in (Aitken & Fisher 1994)). Similarly, an increase in membrane fluidity is necessary to a proper sperm-oocyte fusion. This alteration begins during capacitation and acrosome reaction, in which RS inhibit the activity of phosphotyrosine phosphatases and consequently prevent the dephosphorylation and deactivation of PLA_2. This enzyme cleaves the secondary fatty acid from the glycerol backbone of the phospholipids, which consequently leads to an increase in membrane fluidity (Kothari et al. 2010). In relation to sperm motility, which is a fundamental indicator of sperm quality, RS, namely NO, leads to the activation of soluble guanylyl cyclase

(sGC) and subsequently to the synthesis of cyclic guanosine monophosphate (cGMP) and to the activation of cGMP-dependent protein kinases. Furthermore, a recent study demonstrated that NO increases energy production in mitochondria, which improves sperm motility (Buzadzic et al. 2014).

However, when excessive amounts of RS are produced and overcomes the antioxidant defense mechanisms, OS occurs and leads to pathological defects **Figure 4**). Spermatozoa are susceptible to OS since their plasma membrane is enriched in polyunsaturated fatty acids (PUFA) and their cytoplasm contains low amounts of antioxidant enzymes (Aitken & Krausz 2001; Agarwal & Saleh 2002). Lipids are the main molecules responsible for membrane fluidity and permeability, as well as, for the regulation of polarized migration of sperm surface antigens during developmental processes and the interaction with oocyte.

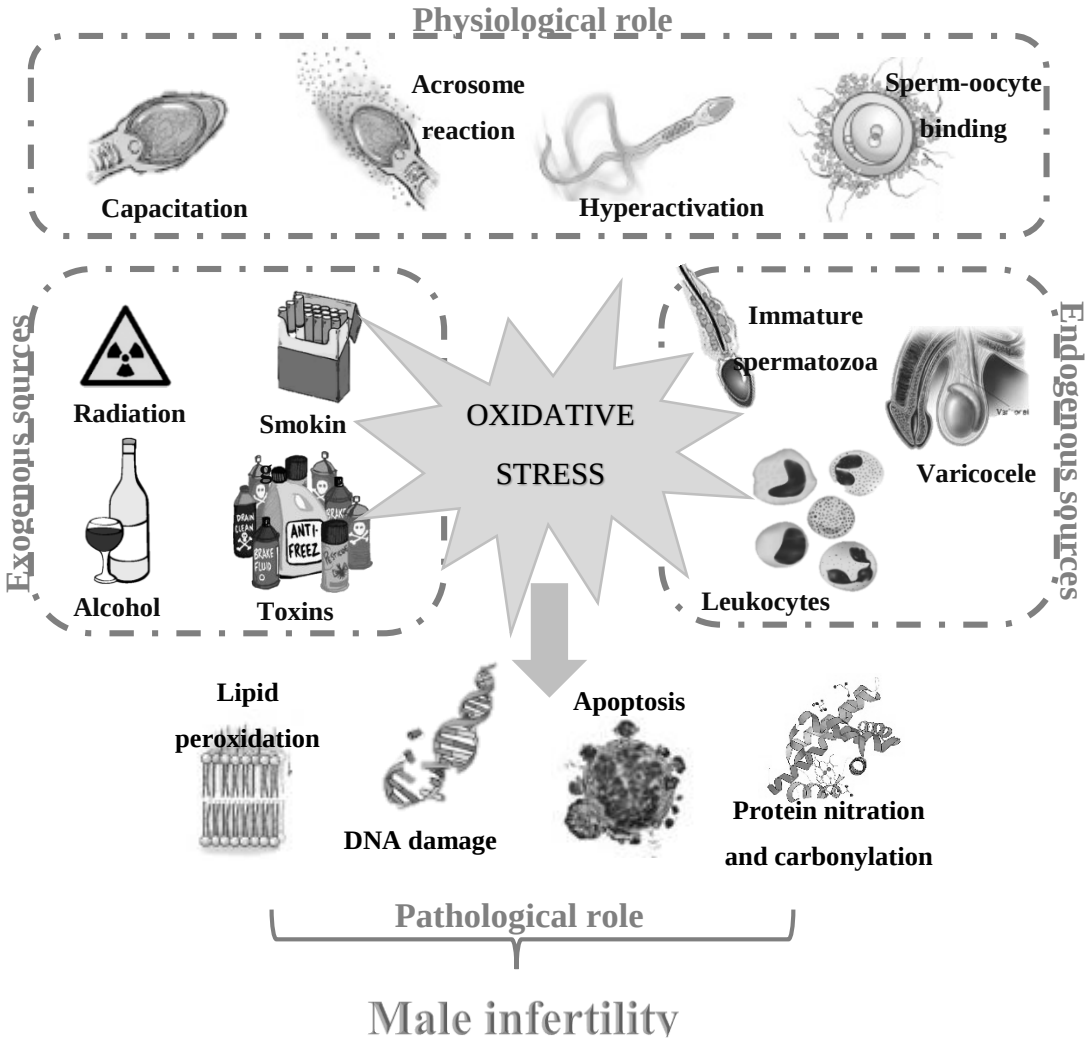

Figure 4 - Main causes (exogenous and endogenous) of oxidative stress and the pathological and physiological effects in sperm cells that may lead to male infertility (adapted from Agarwal

et al. 2014). In low amounts, RS play an important physiological role, namely in the fertilization process. However, when excessive amounts of RS are produced and overcomes the antioxidant defense mechanisms, OS occurs and leads to pathological defects. Spermatozoa are susceptible to OS since their plasma membrane is enriched in PUFA and their cytoplasm contains low amounts of antioxidant enzymes. **RS:** reactive species; **PUFA**: polyunsaturated fatty acids.

Docosahexaenoic acid (DHA) is the major polyunsaturated fatty acid (108 nmol/10^8 cells) present in sperm plasma membrane and is thought to play an important role in the regulation of spermatogenesis and sperm membrane fluidity (Sanocka & Kurpisz 2004). Lipid peroxidation cascade initiates with hydroxyl radical ($OH^{\cdot}$), which results from the reaction between $O_2^{\cdot-}$ and H_2O_2 in the presence of transition metals, such as iron or copper (Aitken & Fisher 1994). Lipid peroxidation involves three major steps: initiation, propagation and termination. In the initiation step the abstraction of a hydrogen atom from an unsaturated fatty acid occurs, leading to the formation of RS. In turn, RS attack fatty acid chains to produce lipid radicals, which can rapidly react with oxygen to form a lipid peroxyl radical (propagation step). Finally, in the final step of lipid peroxidation, peroxyl radicals react with other radicals to form an end product named malondialdehyde (MDA) (Agarwal, Virk, et al. 2014). MDA is widely used as biomarker for lipid peroxidation in conjugation with thiobarbituric acid (TBA), producing a pink-colored chromogen, which is detected with a spectrophotometer. Other aldehyde produced in large amounts is the 4-hydroxynonenal (4-HNE), which is considered as one of the major toxic products, since it rapidly reacts with thiols and amino groups (Sanocka & Kurpisz 2004; Ayala et al. 2014).

Since lipid peroxidation leads to alterations in membrane integrity and fluidity, sperm motility is affected because membrane functions that are critical to flagellar movement are disrupted. Membrane Ca^{2+}/Mg^{2+} ATPase activity is also affected due to the decrease in the membrane fluidity, which consequently also leads to a decrease in sperm motility (Aitken et al. 2010). H_2O_2 is permeable and passes through the membrane, leading to the inhibition of certain enzymes, such as G6PDH. This inhibition leads to a decrease in the NADPH levels and a simultaneous accumulation of GSSG, which causes a decrease in antioxidant defenses and consequently lipid peroxidation in sperm membrane (Agarwal & Saleh 2002).

The presence of high amounts of RS also leads to detrimental alterations in sperm nuclear and mitochondrial DNA. RS attack DNA bases, specially guanine and phosphodiester groups, leading to the destabilization of DNA and consequently to its fragmentation (Gharagozloo &

Aitken 2011). During spermiogenesis, histones are replaced by protamines to form a highly compacted structure, thus protecting sperm chromatin from oxidative damage. However, when a defective compaction and an incomplete protamination occur, sperm chromatin becomes vulnerable to OS (Agarwal, Virk, et al. 2014). Furthermore, sperm DNA is more susceptible to damage because P2 protamines contain less cysteine residues and fewer disulfide crosslinks (Schulte et al. 2010). RS also induce the activation of caspases, which cause indirect DNA damage through endonucleases activation (Wright et al. 2014). However, since sperm midpiece is physically separated from nucleus, endonucleases, activated during apoptosis, remain in the midpiece and do not enter the nucleus. Thus, only RS produced in mitochondria have the ability to induce DNA damage and consequently apoptosis (Aitken et al. 2014). Mitochondrial DNA (mtDNA) is also damaged by RS, decreasing ATP production necessary for sperm motility and thus reducing fertility (Wright et al. 2014).

Several assays have been used to assess the genetic material integrity, which is an important component of reproductive outcome (Agarwal, Virk, et al. 2014). 8-hydroxydeoxyguanosine (8-OHdG) is one of the products of DNA oxidation and is widely used as biomarker for DNA damage. Other strategies used to assess sperm DNA integrity include terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), sperm chromatin structure assay (SCSA), among others (Schulte et al. 2010). To protect DNA from OS, spermatozoa possess DNA repair strategies, such as 8-oxoguanine glycosylase 1 (OGG1), which is found in sperm nucleus. This enzyme cuts 8-OHdG from the DNA and releases it to extracellular space, producing an abasic site. As spermatozoa do not have enzymes to insert a new base, they carry the abasic sites to the oocyte, where the repair process will be concluded (Aitken et al. 2014).

Proteins are also affected by RS. Protein carbonylation occurs in protein side chains, mainly in proline, arginine, lysine and threonine residues, and is used as a marker for protein oxidation. Carbonyl groups (ketones and aldehydes) are introduced in proteins when they are oxidized by RS, but can also be produced by a secondary reaction with products of lipid peroxidation, such MDA and 4-HNE (Dalle-Donne et al. 2003). Other protein modification that occurs due to OS is nitration. The nitration is the addition of a NO_2 group to protein residues in the presence of peroxynitrite, which results in the formation of 3-nitrotyrosine (3-NT) (Vignini et al. 2006). Protein nitration may lead to alterations in protein function and structure, which consequently may result in a decrease in sperm motility (Morielli & O'Flaherty 2014), however a study demonstrated that low concentrations are important for the induction of sperm capacitation (Herrero et al. 2001).

To fight the negative effects of RS and protect the cells, male reproductive system and sperm cells have a set of antioxidant defense mechanisms (**Figure 5**), which include both enzymatic and non-enzymatic molecules (Walczak-Jedrzejowska et al. 2013). In the testis, in response to OS, the translation of mRNA species of superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) occurs, being mediated by NF-kB. Since the major RS produced in germ cells and spermatozoa is $O_2^{\cdot-}$, SOD rapidly converts this free radical into H_2O_2 to prevent the formation of the extremely reactive hydroxyl radical. Due to the importance of SOD, the testis contain three isoforms of this enzyme: the cytosolic Cu/Zn SOD, or SOD1, the mitochondrial Fe/Mn SOD, or SOD2, and an uncommon form of extracellular SOD, named SOD3 or SOD-Ex, which is mainly produced by Sertoli cells. The cytokine interleukin-1α is released by germ cells to stimulate Sertoli cells to secrete SOD3 (Aitken & Roman 2008). Spermatozoa also have these three forms of SOD; however, the amounts of SOD2 and SOD3 are insignificant and, therefore, SOD1 seems to play a major role in the protection against $O_2^{\cdot-}$ (O'Flaherty 2014).

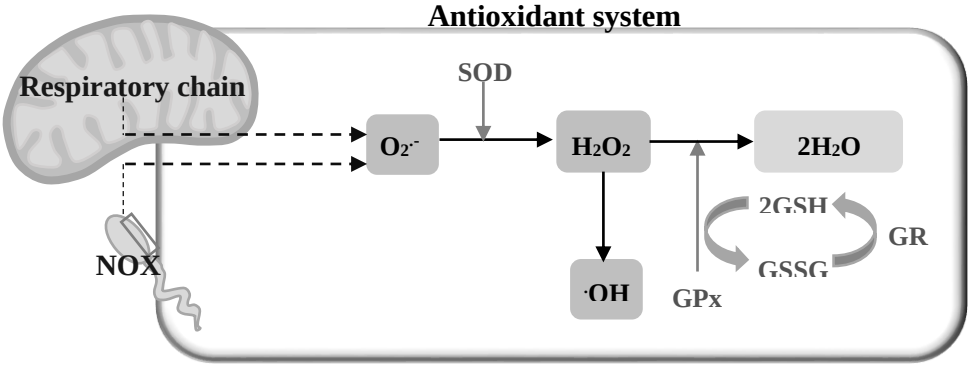

Figure 5 - Generation of RS and activity of antioxidant enzymes. $O_2^{\cdot-}$ is mainly produced in electron transport chain, localized in mitochondria, and by NADPH oxidases presented in plasmatic membrane. SOD rapidly converts $O_2^{\cdot-}$ into H_2O_2 to prevent the formation of the extremely reactive hydroxyl radical. Subsequently, H_2O_2 are converted into H_2O by GPx. **NOX**: NADPH oxidase; **$O_2^{\cdot-}$**: superoxide anion; **SOD**: superoxide dismutase; **H_2O_2**: hydrogen peroxide; **·OH**: hydroxide anion; **GPx**: glutathione peroxidase; **GSH**: reduced glutathione; **GSSG**: oxidized glutathione; **GR**: glutathione reductase.

In turn, after the formation of H_2O_2 by SOD, it is necessary to remove this compound because it is more stable and highly toxic to cells. Catalase (CAT) is responsible for the degradation of H_2O_2 into water and oxygen. This enzyme is found in negligible amounts and consequently does not play an important role in the antioxidant defense of the spermatozoa

(Aitken & Roman 2008; O'Flaherty 2014); however, it is found in seminal plasma, originated from prostate (Walczak-Jedrzejowska et al. 2013). Other enzyme capable of decomposing H_2O_2 and also other organic peroxidized compounds is GPx. Glutathione peroxidase family englobes eight classes [selenium- (Se-) dependent (GPx1 to GPx4) and Se-independent (GPx5 to Gpx8) GPxs], which are codified by different genes. Glutathione (GSH) is used as an electron donor by GPx, which is then recycled by glutathione reductase (GR) in the presence of NADPH (Chabory et al. 2010; O'Flaherty 2014). GPx4, also named phospholipid hydroperoxide GPx, is highly expressed in testis, mainly in germ and Leydig cells (Aitken & Roman 2008). Two important isoforms of GPx4 are found in testis and spermatozoa: a nuclear (snGPx4) and a mitochondrial (mtGPx4) isoform (Chabory et al. 2010). snGPx4 is involved in the oxidation of thiols groups of protamines during sperm chromatin formation in spermiogenesis. This isoform uses protamine cysteine residues as reducing agent instead of GSH (Chabory et al. 2010). In testis, mtGPx4 is an active peroxidase mainly found in Leydig cells and round spermatids (Boitani & Puglisi 2008; Guerriero et al. 2014). However, in spermatozoa, about 50% of mtGPx4 is found in mitochondrial helix in midpiece and is transformed into a structural protein involved in the normal formation of mitochondrial sheath (O'Flaherty 2014). This isoform loses its solubility and enzymatic property but it is still unknown if it can recover its enzymatic function (Chabory et al. 2010). Relatively to others GPxs, testis, spermatozoa and seminal plasma lack GPx2, GPx3 and GPx5. However, GPx5 is only expressed in caput region of epididymis and associates with sperm plasma membrane in the region of acrosome, protecting this structure from peroxidative damage (Taylor et al. 2013). GST is involved in the detoxification of peroxidized lipids and xenobiotics (Aitken & Roman 2008).

In addition to enzymatic antioxidants, testis and spermatozoa are enriched in low molecular molecules with non-enzymatic activity, which help to neutralize and eliminate RS: GSH, coenzyme Q10, vitamins A, E, C and B complex, minerals, and others (Walczak-Jedrzejowska et al. 2013).

1.3. Lifestyle impact on human

1.3.1. Organ systems

A variety of lifestyle or health related habits, such alcohol and tobacco consumption, can have a major impact on a person's health. Tobacco smoke is one of the major causes of morbidity and mortality and it consists of gases, vaporized liquids and particles. The main components that negatively affect health are nicotine and carbon monoxide (Mostafa 2010; Taha et al. 2012), but it also contains recognized carcinogens and mutagens, such as radioactive polonium, cadmium (Cd), lead (Pb) and benzo-(*a*)pyrene (BaP) (Arabi & Moshtaghi 2005). Alcohol consumption is a psychoactive substance that results in approximately 2,5 million deaths each year and leads to adverse health problems and social consequence (WHO 2011).

Many lifestyle factors, such as alcohol and tobacco consumption, are known to be associated with several pathologies, namely cirrhosis and lung cancer, respectively, but also negatively affect other body systems (van Dam et al. 2008). The effects of these lifestyle factors on organ systems are described on **Table 2**.

Table 2 – Effect of alcohol and tobacco consumption on human different targets.

Lifestyle factor	Target	Effect	References
Smoking	Fetus	Limit oxygen distribution; Intrauterine anoxia; Imped access to nutrients; Reduced head growth; Behavioural changes (↑ DNA methylation of BDNF gene);	(Kamer et al. 2014; Ekblad et al. 2015)
	Brain	Alter dopamine release; Affect acetylcholinergic and serotonin receptors;	(Kamer et al. 2014; Ekblad et al. 2015)
	Feminine reproductive system	↓ inhibin B levels; ↓ antral follicle count; Premature ovarian failure; Earlier onset of menopause;	(Waylen et al. 2010)

	Lung	Cancer: ↑ lung carcinogen (NNK); Activate oncogenes (e.g. RAS) and induce mutations on tumor suppressors genes (e.g. TP53); ↑ ATP levels in bronchoalveolar lavage fluid (inducing the release of IL-8 and, consequently, inflammation);	(Hecht et al. 2000; Hecht 2002; Marriott et al. 2012; Belchamber et al. 2014; Gibbons et al. 2015)
	Heart	↑ release of noradrenaline and adrenaline: ↑heart rate and blood pressure;	(Middlekauff et al. 2014; Yan & D'Ruiz 2015)
	Kidney	Renal carcinoma: Inhibition of CYP2A6;	(Cooper 2006)
	Bladder	↑ urinary frequency and urgency;	(Tähtinen et al. 2011; Burgio et al. 2013)
Alcohol	Fetus	Crosses the placenta: Disruption of normal hormonal interactions between the mother and the fetus;	(Gabriel et al. 1998)
	Brain	Activation of TLR4/NF-κB signaling: ↓ myelin formation; Brain damage; Cognitive dysfunctions;	(Pascual et al. 2015)
	Liver	↑ serum liver enzymes; Hypo- and hypermethylation of genes (e.g. RASSF1, GSTP1, MGMT and CHRNA3); ↓ MAT activity and SAMe biosynthesis; Fibrogenesis: ↑ collagens and structural glycoproteins synthesis; ↑ metalloproteinase MMP-2;	(Varela-Rey et al. 2013; Danielsson et al. 2014; Ceni et al. 2014)
	Bone	Inhibition of the transformation of osteoblasts and the growth of mesenchymal stem cells; Transformation of mesenchymal cells into adipocytes; ↑ serum OPG;	(García-Valdecasas-Campelo et al. 2006; Mikosch 2014)
	Bladder	↑ urinary frequency and urgency	(Burgio et al. 2013)
	Kidney	Lipid peroxidation of the membrane of renal tubules; Interference with antioxidant system.	(Ozbek 2012)

1.3.2. Male infertility

The alterations in semen quality have been studied due to the negative effects of many lifestyle factors in the male reproductive system. Behavioral exposure to various substances, such as alcohol, tobacco smoking and others, are known to affect spermatogenesis and, consequently, sperm function (Barazani et al. 2014).

The detection of many components of tobacco smoke in seminal plasma of smokers, such as nicotine, cotinine (the major metabolite of nicotine), Cd, Pb, among others, suggests that these compounds cross the BTB and create an adverse environment for spermatozoa (Sepaniak et al. 2006; Chohan & Badawy 2010). Some of these compounds, such Cd, when ingested, enter in the testis through Zinc-regulated *transporters*, Iron-regulated *transporter*-like Proteins (ZIP) transporters SLC39A8 and SLC39A14 (Cheng & Mruk 2012). The mechanisms of DNA repair only occur in early stages of spermatogenesis (spermatocytes and early spermatids), which consequently leads to the accumulation of DNA damages in mature spermatids and spermatozoa (Horak et al. 2003). It is known that Cd disrupts the BTB, since it has the ability to mimic other divalent cations, such as calcium (Ca^{2+}), interfering with cell adhesion molecules, which are present in this barrier and are Ca^{2+}-dependent proteins (**Figure 6**) (Cheng & Mruk 2012). Furthermore, this "ionic imitation" may interfere with important cellular transport processes. Cd and BaP also activate aryl hydrocarbon (Ah) receptor. When the ligand binds to this receptor, this complex is translocated to the nucleus, leading to the transcription of genes for phase 1 (cytochrome P450) enzymes. Subsequently, BaP is metabolized, generating a procarcinogenic compound, designated BPDE. This compound creates modifications in DNA bases, which were observed in animals exposed to BaP (Revel et al. 2001; Sharpe 2010).

Consequently, the normal production of spermatozoa and their function will be affected. Studies in animal models have demonstrated that exposure to the components of tobacco smoke results in the atrophy and degeneration of seminiferous tubules, vacuolization of Sertoli cells and decrease in Leydig and Sertoli cell numbers (Ahmadnia et al. 2007; Nesseim et al. 2011; Ahmed et al. 2013). Many studies in men with behavioral exposure have reported negative impact on sperm parameters, resulting in the decrease of sperm volume, concentration, motility, viability and normal morphology (Sofikitis et al. 1996; Mak et al. 2000; Arabi & Moshtaghi 2005; Kiziler et al. 2007; Kumosani et al. 2008; Liu et al. 2010; Tawadrous et al. 2011; Ghaffari & Rostami 2012; Ghaffari & Rostami 2013; Hamad et al. 2014), as well as, an increase of round cells in the ejaculate (Trummer et al. 2002). However, some studies remain controversial (Sepaniak et al. 2006; Davar et al. 2012). Alterations in oxygen supply to the testis due to tobacco

consumption may also be a mechanism that negatively affects the normal spermatogenic process (Sharpe 2010).

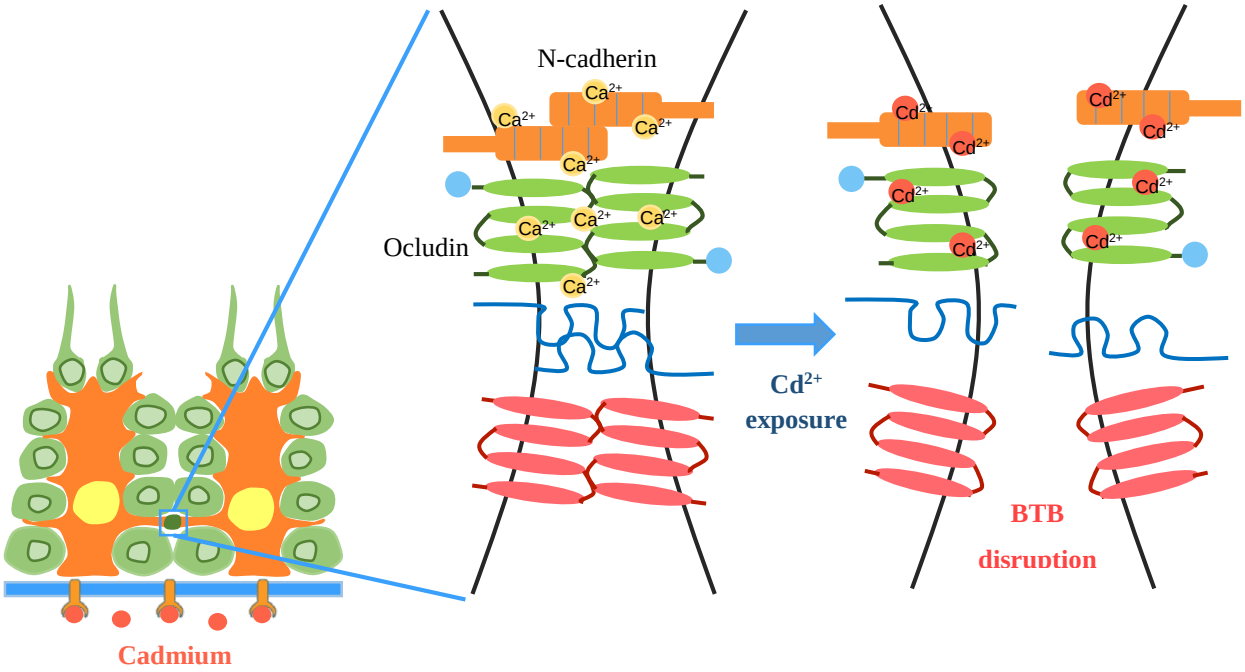

Figure 6 - Schematic drawing illustrating the effect of cadmium exposure on junction restructuring in the blood-testis barrier. Cd disrupts the BTB, since it has the ability to mimic other divalent cations, such as Ca^{2+}, interfering with cell adhesion molecules (ocludin and N-cadherin), which are Ca^{2+}-dependent proteins. This "ionic imitation" may interfere with important cellular transport processes. **Cd^{2+}**: cadmium; **Ca^{2+}**: calcium; **BTB**: blood-testis barrier.

Kumosani et al. (2008) showed that smokers have increased concentrations of Cd in seminal plasma and observed a decrease in Ca^{2+}-ATPase activity, which is required for Ca^{2+} homeostasis and signaling. Epididymal and prostate function is also affected, since smoking leads to a reduction in α-glucosidase and zinc concentration in seminal plasma (Sofikitis et al. 1996; Liu et al. 2010), respectively, which are compounds used as biomarkers of these glands secretory activity (Andrade-Rocha 2003). As epididymis is important for sperm maturation, and consequently to motility acquisition, the alterations caused by tobacco smoking may lead to a decrease in sperm motility and fertilizing capacity (Sofikitis et al. 1996).

Studies concerning the effect of tobacco smoking on the pituitary-gonadal axis have reported conflicting findings. Testosterone levels were found decreased in smokers (Sofikitis et al. 1996), while other studies demonstrated an increase in free and total testosterone due to

tobacco smoking (English et al. 2001; Trummer et al. 2002; Blanco-Muñoz et al. 2012). In the same way, analysis of prolactin levels demonstrated contradictory results (Trummer et al. 2002; Blanco-Muñoz et al. 2012). LH levels were found increased in male smokers, while other hormones levels, such as FSH, inhibin B and estradiol, remained unchanged (English et al. 2001; Halmenschlager et al. 2009; Blanco-Muñoz et al. 2012).

Many toxics are responsible for a defective DNA compaction, leaving DNA susceptible to oxidative attack (Grootegoed et al. 2000; Gharagozloo & Aitken 2011). A recent study demonstrated that smokers have alterations in histone to protamine transition, observed by a higher proportion of histone H2B, leading to an abnormal chromatin structure and consequently to infertility (Hamad et al. 2014). Smoking also seems to induce apoptosis, detected by the presence of Smac/DIABLO, indicating an initiation by mitochondrial pathway. OS induced by smoking exposure may be the cause for the apoptotic process initiation, perturbing the mitochondrial integrity and consequently leading to the release of cytochrome *c* and Smac/DIABLO (Ramalho-Santos et al. 2009; Tawadrous et al. 2011). An increase in caspase-9 in seminal plasma was also verified in smokers and is negatively correlated with sperm motility, since apoptosis leads to mitochondrial alterations (Tawadrous et al. 2011). One of the features of apoptosis is the fragmentation of DNA and the externalization of phosphatidylserine (PS) in the plasma membrane (Amaral, Lourenço, et al. 2013). Many studies have demonstrated that tobacco smoking increases the membrane PS translocation, the apoptotic population (Belcheva et al. 2004) and the fragmentation of sperm DNA (Sofikitis et al. 1996; Sepaniak et al. 2006; Tawadrous et al. 2011; Taha et al. 2012).

Other mechanism of smoking toxicity is due to the incitation of OS, via changes in cytokines, leading to the activation of TGF-β2, -β3 and p38 MAPK in the testis (Thompson & Bannigan 2008; Cheng & Mruk 2012). The increase in OS also occurs due to the binding of Cd to sulfhydryl-containing proteins, leading to their inactivation (Siu et al. 2009). OS was also verified in smokers by the presence of high levels of RS in seminal plasma (Kiziler et al. 2007; Taha et al. 2012) and low levels of antioxidant enzymes (Kiziler et al. 2007; Elshal et al. 2009). In addition, smokers showed increased levels of leukocytes (Trummer et al. 2002), which are the main sources of RS in seminal plasma, leading consequently to biomolecules damage (Schulte et al. 2010). The excessive production of RS, induced by smoking, leads to lipid peroxidation, verified by the presence of high levels of MDA (Arabi & Moshtaghi 2005; Kiziler et al. 2007; Ghaffari & Rostami 2012; Hamad et al. 2014) and thiobarbituric acid reactive substance (TBARS) (Elshal et al. 2009), and to protein oxidation, by the increase in protein carbonyls (Kiziler et al. 2007).

A recent study demonstrated that tobacco smoke also directly inhibit both neuronal and endothelial isoforms of nitric oxide synthase (NOS), impairing NOS-mediated vasodilation of penile arterioles. In addition, $O_2^{\cdot -}$ from tobacco smoke directly degrade NO in the corpora cavernosa. These effects lead to cardiovascular dysfunction and consequently to erectile dysfunction (Kovac et al. 2014).

Other lifestyle factor responsible for adverse effects in male reproductive system is alcohol consumption (**Figure 7**), due to the induction of OS. When alcohol enters in the organism, it is converted to acetaldehyde, a highly toxic and reactive compound, by alcohol dehydrogenase (ADH) and subsequently into acetate by aldehyde dehydrogenase (ALDH). In each one of these reactions a molecule of NADH is formed leading to an increase in the activity of the respiratory chain, and consequently to an increase in RS production (Wu et al. 2006). Furthermore, acetaldehyde interferes with the testosterone production through the inhibition of protein kinase C, involved in this process (Emanuele & Emanuele 2001), and may interact with proteins and lipids, increasing RS generation (Talebi et al. 2011). The excessive production of RS may also be due to an increase in the activity of CYP2E1. RS generation is higher in the testis, due to the production of steroids hormones, because its formation uses oxygen, which results in free radical generation. RS also interfere with cholesterol transport and the function of steroidogenic enzymes, inhibiting steroidogenesis (Wu et al. 2006; Jana et al. 2010). Beta-endorphin, an opioid, seems to increase due to alcohol consumption leading to an increase in apoptosis and a suppression of testosterone synthesis and release in the testis (Emanuele & Emanuele 2001). Furthermore, alcohol may lead to a diminution of the secretion of hypothalamic GnRH and the cleavage of GnRH precursor into a functionally active hormone, inhibiting consequently the hypothalamic-pituitary-gonadal (HPG) axis. Alcohol may also have a direct inhibitory effect on pituitary gland, constraining LH production and secretion (Emanuele & Emanuele 2001; Barazani et al. 2014).

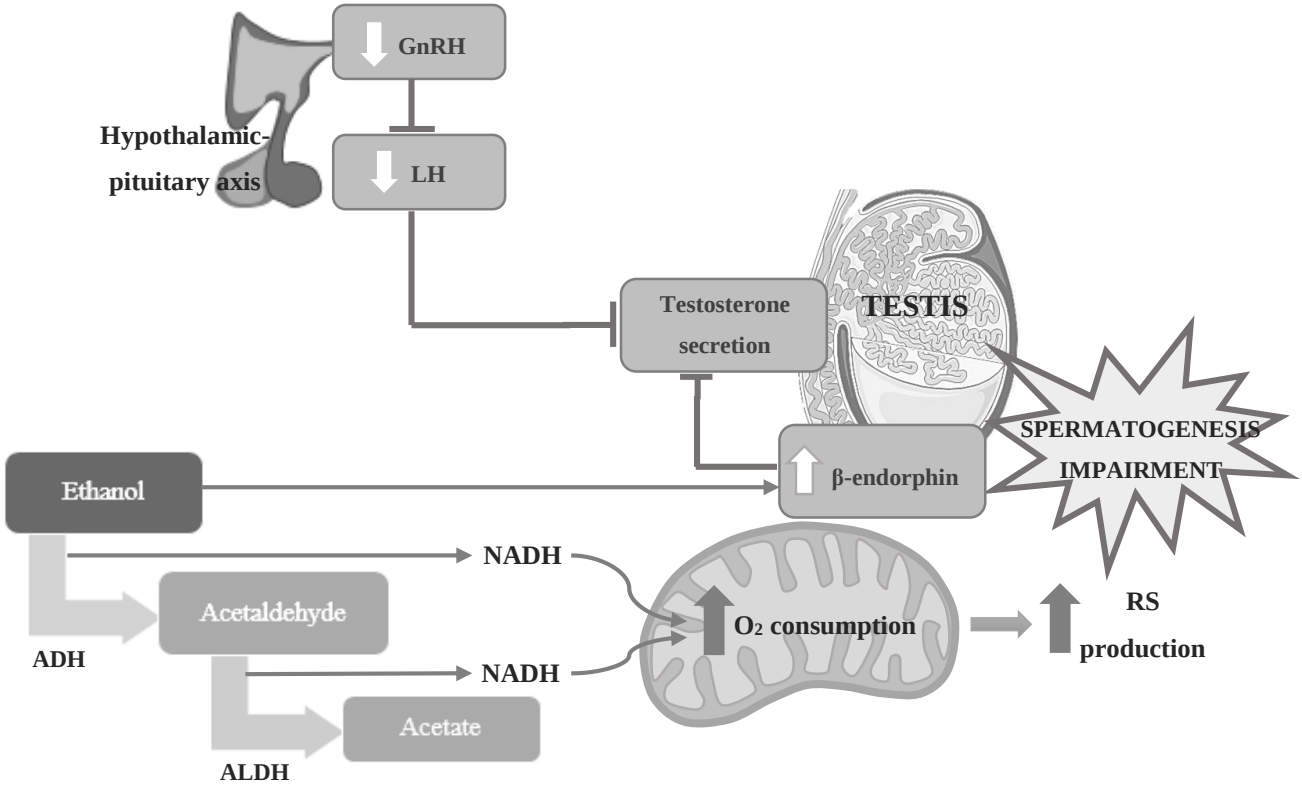

Figure 7 - Action mechanism of alcohol consumption on male reproductive system. When alcohol enters in the organism, it is converted to acetaldehyde by ADH and subsequently into acetate by ALDH. In each one of these reactions a molecule of NADH is formed leading to an increase in the activity of the respiratory chain, and consequently to an increase in RS production. Beta-endorphin seems to increase due to alcohol consumption leading to a suppression of testosterone synthesis and release in the testis. Furthermore, alcohol may lead to a diminution of the secretion of hypothalamic GnRH and may also have a direct inhibitory effect on pituitary gland, constraining LH production and secretion. Figures were produced using Servier Medical Art. **GnRH:** Gonadotropin-releasing hormone; **LH:** Luteinizing hormone; **ADH:** Alcohol dehydrogenase; **ALDH:** aldehyde dehydrogenase; **NADH:** nicotinamide adenine dinucleotide; **RS:** reactive species.

Studies about the impact of alcohol consumption on male reproductive system have demonstrated that alcohol leads to a decrease in testicular weight and impairment of spermatogenesis (Pajarinen et al. 1996), and also a decrease in semen parameters, namely progressive motility, concentration and normal morphology (Hansen et al. 2012; Jensen, Gottschau, et al. 2014; Condorelli et al. 2014); however, contradictory results exist in literature, since no alterations in semen quality were observed by Jensen and colleagues (Jensen, Swan, et al. 2014). Sermondade and coworkers evaluated semen parameters during chronic alcohol

consumption and after withdrawal and they verified that alcohol withdrawal allowed an improvement in semen quality (Sermondade et al. 2010). Additionally, sons of women that consumed alcohol during pregnancy have sperm volume and concentration decreased with increasing prenatal alcohol consumption (La Vignera et al. 2013). Animal studies have also showed that ethanol exposure results in the loss of spermatogenic cells and a decrease in sperm progressive motility. Furthermore, alcohol induces the apoptotic process, showed by the increased expression of Fas/Fas-L system, p53, Bax, Bcl-2, caspase-3 and caspase-8 (Jang et al. 2002; Jana et al. 2010; Talebi et al. 2011). TUNEL assay used in testicular sections revealed that spermatocytes and spermatids are the main germ cells undergoing apoptosis due to alcohol exposure (Jana et al. 2010).

In relation to the effects on pituitary-gonadal axis, results are not consistent. A decrease in testosterone levels was observed by Condorelli et al. (2014), but an increase in testosterone and free testosterone was detected by Jensen, Gottschau, et al. (2014). An increase in FSH, LH and estradiol and a decrease in sex hormone-binding globulin (SHBG) levels were also observed after alcohol consumption (Hansen et al. 2012; Condorelli et al. 2014; Jensen, Gottschau, et al. 2014). However, in another study, no differences in total testosterone, FSH, LH and inhibin B were detected (Hansen et al. 2012). In mice submitted to an ethanol treatment a decrease in the expression of three important enzymes involved in testosterone biosynthesis (testicular StAR, 3β-HSD and 17β-HSD) was observed, and the results were consistent with the diminution of testosterone due to alcohol consumption (Jana et al. 2010).

The contradictory results may be explained by the polymorphisms that exist in ethanol-metabolism genes (ADH and ALDH2), which may lead to a different predisposition to alcohol and effects on reproductive function. Furthermore, glutathione S-transferase M1 locus may also influence the consequences of alcohol in spermatogenesis (Sermondade et al. 2010).

Our laboratory also studied the effect of alcohol and tobacco consumption during the academic week festivities and observed a diminution in sperm quality, such as a decrease in sperm concentration, progressive and non-progressive motility and an increase in abnormal sperm morphology, as well as in the expression of cleaved PARP, which is a biomarker of apoptosis (Ferreira et al. 2012). Other study also confirmed the negative effects of smoking and alcohol consumption in semen parameters, such as semen volume and percentage of degenerated spermatozoa, and DNA fragmentation, which have serious consequences on male fertility (Anifandis et al. 2014). However, a study determined the effect of smoking and drinking habits

on semen parameters and observed that these lifestyles did not affect semen quality or pregnancy outcome (de Jong et al. 2014).

Since infertility have been mainly attributed to OS and many lifestyle alterations are known to incite OS, leading to the impairment of cells function, it is important to know how the production of spermatozoa is affected and the subsequently implications for the function of these cells. In this study, and because in the majority of existing studies the antioxidant capacity of the seminal fluid was evaluated, we will focus in the total antioxidant capacity of the sperm cells and the damage caused by OS in the sperm proteins.

2. Aims

OS is considered one of the main causes of male infertility, being a significant contributing factor in 30–80% of the cases (Esteves & Agarwal 2011). Over the last decades, the progressive changes in lifestyle habits have led to negative impacts on reproductive health. Although sperm cells are susceptible to OS due to the presence of polyunsaturated fatty acids, they have an antioxidant defense system, which includes mainly superoxide dismutase (SOD) and the glutathione–peroxidase–reductase system (Taylor 2001). Since the induction of OS leads to a disequilibrium on oxidative balance and consequently to the damage of spermatozoa biomolecules, the goals of this thesis were:

- to analyze the impact of lifestyle changes in the semen analysis parameters, according to WHO guidelines;
- to assess the immunolocalization of the antioxidant enzymes SOD1, GPx1 and GPx4 in the sperm cells, using normal fresh semen samples;
- to analyze the impact of the lifestyle alterations in the antioxidant capacity and in the OS-induced protein damage of sperm cells during the academic festivities week;
- to evaluate the correlation between the OS parameters analyzed and the seminal parameters studied.

3. Methods

3.1. Semen Samples

Semen samples used were acquired during the "*Para o Frasco*" study to evaluate the influence of acute lifestyle changes during the academic festivities, such as the consumption of alcohol, tobacco and drugs, in the male fertility. The samples were given by young male volunteers, in reproductive age (more than 18 years), at University of Aveiro, during three defined different moments: the first moment (time point 1 – TP1) was before the academic week, the second moment (time point 2 – TP2) was one week after the academic festivities and the third moment (time point 3 – TP3) was around three months after this festivity.

When the volunteers delivered their samples, in TP1, they answered to a questionnaire (questionnaire 1- see in **Appendix 8.1.1**) where the habitual drug, alcohol and tobacco consumption as well as their sexual abstinence and the history of diseases were asked. At the second sample delivery, the volunteers answered to a new questionnaire (questionnaire 2 – see in **Appendix 8.1.2**) where the questions were identical, but the consumption questions were about the academic festivities. They also answered to a questionnaire at TP3 about their consumption during the last three months (questionnaire 3 – see in **Appendix 8.1.3**). A number was attributed to each volunteer, followed by the number of the corresponding TP. For example, 1.1 and 1.2 corresponds to the semen sample of volunteer number 1 in TP1 and TP2, respectively. The information of each volunteer are presented in **Appendix 8.3, 8.4, 8.5** and **8.6**.

Sample used in this study were already processed and lysed (**Figure 8**). Then, it was performed a protein assay, evaluated the TAS as well as the presence of SOD1, GPx4 and carbonyl and 3-NT groups. Immunocytochemistry analysis was performed with fresh human sperm cells.

The samples of semen were collected to a sterile recipient by masturbation according to the WHO recommendations. After the collection, the samples were maintained at room temperature (RT) during a maximum of one hour. Then, the samples were allowed to liquefy by incubation at 37 ºC for approximately 30 minutes.

After the liquefaction, the basic semen analysis was performed, according to WHO guidelines.

Semen samples for western and slot blot analysis were washed with PBS (1:2 ratio). The seminal plasma and spermatozoa were separated by a centrifugation during 5 minutes, 400 g at 4 ºC. After this step, the supernatant (seminal plasma) was frozen at -20 ºC and stored. The pellet

obtained during the centrifugation, which corresponds to the spermatozoa, was washed and then centrifuged twice with PBS. The pellet was frozen at -20 ºC with 1% SDS.

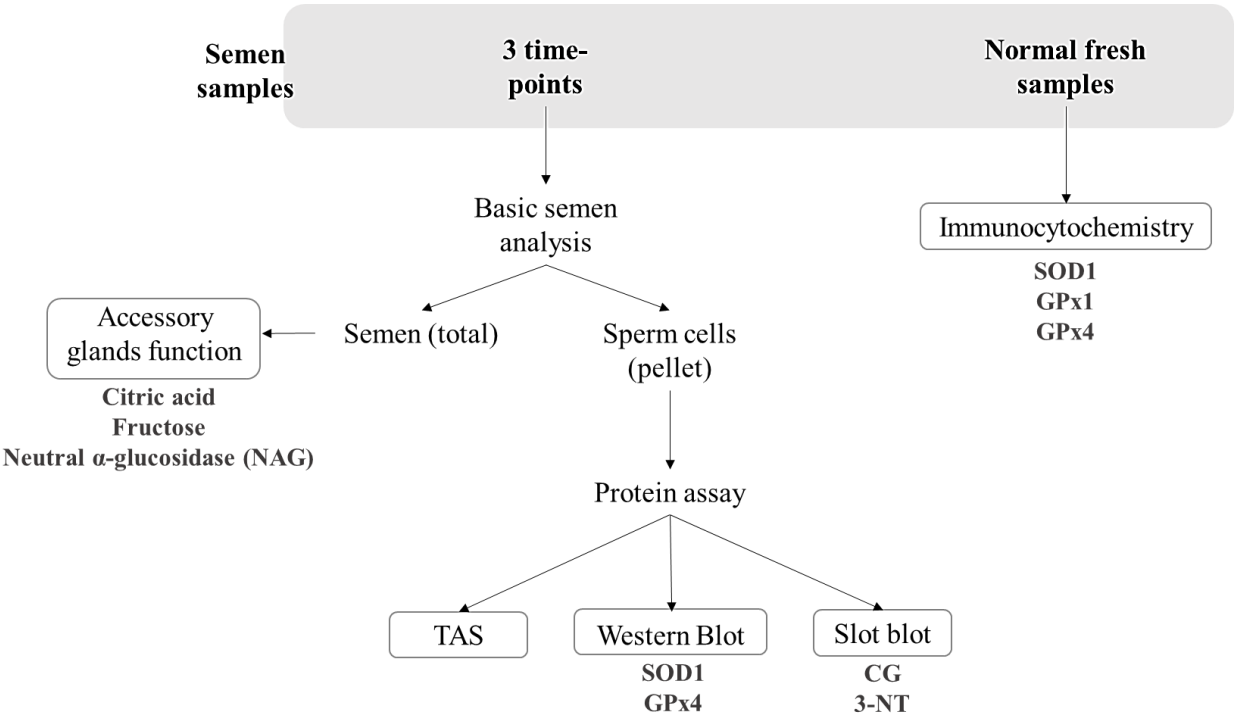

Figure 8 - Sample preparation and assays performed in this work.

3.2. Seminal Analysis

3.2.1. Macroscopic Examination

The macroscopic examination began soon after liquefaction, to prevent dehydration or changes in temperature that will affect the semen quality.

After the ejaculation, the semen is typically a semisolid coagulated mass. After a few minutes at RT, the semen initiates the liquefaction that leads to a more homogeneous and quite watery sample. The process of liquefaction usually takes 15 minutes at RT; however, in some cases it can take up to 60 minutes or even more.

After liquefaction, the viscosity of the sample was evaluated. This parameter can be analyzed by aspirating the sample using a micropipette and allowing the semen to drop by gravity observing the length of any thread. When the viscosity is normal, the sample is

dispensed by micropipettes in small discrete drops. An abnormal sample forms drops with a thread with more than 2 cm long.

Regarding appearance, a normal sample shows a homogeneous and grey-opalescent appearance. When the semen sample has a low sperm concentration, it appears less opaque. The color of the sample may also have some alterations: when the red blood cells are present, the sample has a red-brown color; the samples of men with jaundice or taking certain vitamins or drugs may show a yellow color.

Finally, the sample was transferred from the container to a centrifuge tube of 15 mL and the volume was read directly from the graduations.

3.2.2. Microscopic Examination

The microscopic examination includes the analysis of motility, concentration and morphology of the spermatozoa. These parameters were performed based on WHO guidelines.

3.2.2.1. Aggregation and Agglutination

Aggregation refers to the adherence of non-motile spermatozoa to each other or even to the adherence between motile spermatozoa and mucus strands, non-sperm cells as well as debris.

In turn, agglutination refers to the adherence between several motile spermatozoa; this interaction might be head to head, tail to tail or mixed. In the majority of the cases, the motility is present with a shaking motion; however, when the sample is highly agglutinated the motion is limited. There are four grades of agglutination:

Grade 1 – less than 10 spermatozoa per agglutinate with many free spermatozoa;
Grade 2 – 10 to 50 spermatozoa per agglutinate with free spermatozoa;
Grade 3 – more than 50 spermatozoa per agglutinate with few free spermatozoa;
Grade 4 – all spermatozoa are interconnected.

3.2.2.2. Concentration

The sperm concentration can be measured without dilution from $2x10^6$ and $50x10^6$ sperm cells/mL. The evaluation was performed in this range to decrease the presence of lecture errors. First, we mixed the liquefied semen sample and put on a glass slide covered with a coverslip to determinate the appropriate dilution (**Table 3**). Then, the appropriate volume of sample was added to a fixative (composed of 5 g of $NaHCO_3$ and 1 mL of 35% (v/v) formalin in 100 mL of distilled water). This mixture was then vortexed for 10 seconds and the improved Neubauer chamber filled with 10 µL of the mixture. The chamber was analyzed at 400x magnification, counting at least 200 spermatozoa. Initially, the central grid (number 5) of one side was assessed. Then, the concentration of spermatozoa per milliliter was determinate using the following formula: C = (N/n) x (1/20) x dilution factor; N is the number of spermatozoa, n is the volume of the total number of rows examined (20 ηL each for grid number 5). Lastly, the total number of spermatozoa in ejaculate was determined by multiplying sperm concentration by the semen volume.

Table 3 - Semen dilutions required for the determination of sperm concentration.

Spermatozoa per 400x field	Dilution	Semen (µL)	Fixative (µL)
>101	1:20	50	950
16-100	1:5	50	200
<15	1:2	50	50

3.2.2.3. Motility

The sperm motility was analyzed as soon as possible after liquefaction, preferably 30 minutes, to limit the deleterious effects of dehydration, changes in pH or temperature. The sperm motility could be assessed on samples with sperm concentrations between $2x10^6$ and $50x10^6$ spermatozoa. To evaluate the sperm motility we first mixed the semen sample, removing immediately an aliquot. Then, we prepared a wet preparation; this preparation must have approximately 20 µm deep, and requires a volume of 10 µL. We proceeded to the slide examination with a phase-contrast optics at x400 magnification. The percentage of the different

motile categories (motile progressive, motile non-progressive and immotile) were assessed in approximately 200 spermatozoa.

3.2.2.4. Morphology

To assess sperm morphology, we prepared a smear of semen and the spermatozoa were then assessed for the percentage of normal vs abnormal forms. The abnormalities of the spermatozoa can refer to head, midpiece and tail defects as well as an excess of residual cytoplasm. A semen smear was prepared by application of 10 μL of semen to the end of the slide. A coverslide was used to pull the drop of semen along the surface of the slide. The slides were then allowed to dry in air before fixation that was performed with methanol 95% during 1 hour. The staining was performed in two steps: the cytoplasm staining was performed with eosin during 10 seconds, conferring a pink coloration; in turn, the nucleus was colored with thiazine for 10 seconds, obtaining a blue coloration.

3.3. Accessory glands function analysis

3.3.1. Measurement of neutral α-glucosidase in seminal plasma

Neutral α-glucosidase (NAG) assay is commercially available (EpiScreen Plus™) and was executed according to the instructions of the manufacturer (FertiPro, Beernem, Belgium).

Briefly, 20 μL of each sample were pipetted into two eppendorf tubes. After adding 130 μL of reaction solution to one reaction vessel and 130 μL of inhibitor solution to another one, vortex and incubate exactly during 2 hours at 37 ºC. Subsequently, incubation was stopped by adding 1 mL of reagent 4 and was vortexed. Finally, 200 μL of all standards/samples were pipetted into a microtiter plate and the absorbance was read at 405 nm.

3.3.2. Measurement of fructose in seminal plasma

The measurement of fructose in seminal plasma is commercially available (Fructose Test™) and was executed according to the instructions of the manufacturer (FertiPro, Beernem, Belgium).

Initially, the semen sample was allowed to liquefy at RT. Seminal plasma (100 µL) and fructose (100 µL) standards were pipetted into separate test tubes. Reagent 1 (0,5 mL) was added to the samples (and standards) and centrifuge for 10 minutes. Afterwards, supernatant/standard (20 µL) was pipetted into an empty eppendorf tube and, sequentially, Reagent 2 (200 µL) and Reagent 3 (indole) (20 µL) was added to each of the tubes. The tubes were sealed and incubated for 30 minutes at 37 °C in a water bath and then 200 µL of Reagent 4 was added. Finally, 200 µL of sample was pipetted into an empty well and read at 470-492 nm.

3.3.3. Measurement of citric acid in seminal plasma

The measurement of citric acid in seminal plasma is commercially available (Citric Acid Test) and was executed according to the instructions of the manufacturer (FertiPro, Beernem, Belgium).

Initially, 100 µL of Reagent 2 was mixed with 100 µL of seminal plasma. After 10 minutes of centrifugation, supernatant (25 µL) were pipetted into an empty well and, subsequently, 200 µL of Reagent 1 was slowly added. Finally, the mixture reaction and the standard were read at 405 nm.

3.4. Protein Assay - Bicinchoninic Acid (BCA) assay

To determine the total concentration of protein of the semen samples, the bicinchoninic acid (BCA) assay was performed, using a kit from Pierce and following the instructions of the manufacturer. This method is based in the reduction of Cu^{2+} to Cu^{+}, which is promoted by proteins in an alkaline medium (the biuret reaction); then, colorimetric detection of Cu^{+} cation is performed using a unique reagent containing bicinchoninic acid. The chelation of two molecules of BCA with one Cu^{+} ion is responsible for the formation of a purple-colored reaction product, which exhibits a strong absorbance at 562 nm that is linear with increasing protein

concentrations over a working range of 20 to 2000 µg/mL. This assay is not an end-point method because the final color continues to develop. However, following incubation, the rate of color development decreases, and that situation allows that a large number of samples can be assayed together.

The samples (3 µL) were prepared to be assayed by adding 22 µL of 1% SDS. To determine the total protein concentration of each sample we prepared the standard protein concentrations (**Table 4**).

Table 4 - Preparation of diluted bovine serum albumin (BSA) standards to obtain the standard curve used in BCA protein assay method.

Standard	BSA (µL)	Protein mass (µg)	1% SDS (µL)	W.R. (µL)
P0	0,0	0,0	25,0	200,0
P1	0,5	1,0	24,5	200,0
P2	1,0	2,0	24,0	200,0
P3	2,5	5,0	22,5	200,0
P4	5,0	10,0	20,0	200,0
P5	10,0	20,0	15,0	200,0

BSA: Bovine Serum Albumine**; SDS:** Sodium Dodecyl Sulfate**; W.R.:** Working Reagent

The stock solution of bovine serum albumin (BSA) used had a concentration of 2 mg/mL. The working reagent (W.R.) solution was prepared by mixing reagent A with reagent B, in a proportion of 50:1. Samples and standards were loaded (25 µL) in the 96-well microplate. Then, 200 µL of W.R. was added to each well and the microplate was incubated during 30 minutes at a temperature of 37ºC. The microplate was then allowed to cool to RT during 5 minutes and the absorbance was measured at 562 nm in a microplate reader (iMark™, BioRad, Sintra, Portugal). Using the standard protein concentrations and their subsequent absorbance, we obtained a standard curve that was used to determine the protein concentration of each sample.

3.5. Total Antioxidant Status (TAS)

The concentration of antioxidants in sperm cells was measured using a commercial test for total antioxidant status (TAS) determination and was executed according to the instructions

of the manufacturer (Randox Laboratories, Crumlin, Northern Ireland). This test is based on the incubation of ABTS [2,2-azino-di-(3-ethylbenzthiazoiine sulphonate)] with a peroxidase (metmyoglobin) and H_2O_2; this incubation is responsible for the production of the radical cation $ABTS^+$, which has a relatively stable blue-green color, measured at 600 nm. The sperm cells contain antioxidants, and the addition of these cells to the reaction mixture causes suppression of the color formation; this suppression is proportional to the antioxidant capacity of the added sample.

In this experiment, first, ddH_2O or standard or sample were mixed with the chromogen (R2). Then, the mixture was brought to 37 ºC and after few minutes, the initial absorbance (A1) was read at 600 nm. Finally, substrate (R3) was added for exactly 3 minutes and the final absorbance (A2) was recorded. After these steps, the calculations were performed as following:

Calculation:

$$A2 - A1 = \Delta A\ of\ sample/standard/blank$$

$$Factor = \frac{conc\ of\ standard}{(\Delta A\ blank - \Delta A\ standard)}$$

$$mmol/l = Factor \times (\Delta A\ blank - \Delta A\ sample)$$

The reference range for human plasma blood is 1,30-1,77 mmol/L, according to manufacturer. However, the manufacturer does not had reference values for sperm cells, and the previous studies performed in this area only analyzed the seminal plasma, with a range from 1,7 to 2,3 mmol/L.

3.5.1. Protocol

Previously, in our laboratory we made a few alterations in the protocol. The modified protocol (**Table 5**) was based in the total protein concentration and the cuvette method was modified to a microplate method with the subsequent scaling down of the volumes. All the samples had a protein concentration of 15 μg in a maximum volume of 5 μL. The samples with a higher concentration were diluted in 1% SDS. The amount of reagents used in this assay was

also decreased. So, we used 5 µL of sample or ddH_2O or standard, 200 µL of chromogen and 40 µL of substrate.

Table 5 - Protocol modified to an equal protein concentration (15 µg).

Reagent	Blank	Standard	Sample
ddH_2O	5 µL	---	---
Standard	---	5 µL	---
Sample	---	---	5 µL
Chromogen (R2)		200 µL	
Substrate (R3)		40 µL	

3.6. Western Blotting

In order to visualize the antioxidant proteins, SOD and GPx4, with molecular weights of approximately 18 and 20 kDa, respectively, we performed 12% SDS-PAGE small gels (**Table 6**). We used β-tubulin with a molecular weight of 50 kDa as a loading control.

Table 6 - Composition of running and stacking gels for SDS-PAGE.

Reagent	Running Gel (12%)	Stacking Gel (3,5%)
H_2O	3,4 mL	1,7 mL
Acrylamide	4 mL	0,3 mL
4x LGB	2,5 mL	---
5x UGB	---	0,5 mL
10% SDS	---	25 µL
10%APS	50 µL	25 µL
100% TEMED	5 µL	2,5 µL

APS: Ammonium Persulfate**; LGB:** Lower Gel Buffer**; SDS:** Sodium Dodecyl Sulfate**; TEMED:** Tetramethylethylenediamine**; UGB:** Upper Gel Buffer

Samples were prepared by adding 1% SDS and 4x LB to a total amount of 50 μg of protein, and run in a 12% gel at 200 V. Afterward, proteins were electrotransferred onto nitrocellulose membranes at 200 mA.

Then, the membrane was carefully removed and a Ponceau coloration was performed. The membrane was then washed with distilled water until the proteins were well defined. To remove the coloration of the membrane, it was destained completely by repeated washing in distilled water.

The resultant membrane was hydrated in 1X tris buffered saline (TBS) for 5 minutes and blocked with a solution of 5% non-fat milk in 1X TBS with tween (TBST) for 1 hour. The membrane was then incubated for 2 hours with the primary antibodies anti-β-tubulin (clone AA2, Cat #05-661, Lot #2398882, monoclonal antibody), anti-SOD1 (clone 6F5, Cat# MABC864, Lot #VP1310030, monoclonal antibody) and anti-GPx4 (Cat #ABC269, Lot #VP1306033, polyclonal antibody). All antibodies were from Merck (Merck Millipore, Darmstadt, Germany) and were diluted 1:1000 in 1x TBS-T/5% low fat milk. After that, the membrane was washed with 1x TBS-T during 10 minutes, 15 minutes and 10 minutes, sequentially. Then, the membrane was incubated for 1 hour with the respective fluorescent secondary antibodies, 926-68071 IRDye 680RD Goat anti-Rabbit and 926-32210 IRDye 800CW Goat anti-Mouse (LI-COR BioSciences), diluted 1:5000 in 1x TBS-T/5% low fat milk: anti-mouse for β-tubulin and SOD1 and anti-rabbit for GPx4. Finally, the membrane was washed as before and scanned in LI-COR's Odyssey Infrared Imaging System (LI-COR Biosciences).

3.7. Carbonyl Group Determination

We performed a slot blot method followed by immunodetection for determination of the carbonyl groups.

The samples were prepared as follows: in a microtube it was pipetted an amount of 30 μg of protein/50 μL of sample. The same volume (50 μL) of 12% SDS was added and the mixture spin-down for 5 seconds. Next, it was added 2 volumes (100 μL) of 20 mM DNPH/10% trifluoroacetic acid (TFA). Then, the samples were incubated for 30 minutes in the dark. Finally, the solution was neutralized with 75 μL of 2M Tris/18% of mercaptoethanol.

To perform the slot blot, the samples were diluted with 1x PBS to a concentration of 2 ηg/μL. The membrane was incubated in 10% methanol for activation, washed in water and posteriorly in 1x TBS. Then, 100 μL of each sample were applied in the slot blot (BioRad Portugal, Sintra, Portugal) and transferred to the nitrocellulose membrane.

The nitrocellulose membrane was blocked for 1 hour in 1x TBS-T/5% low fat milk. Next, the rabbit antibody anti-DNP, clone 9H8.1, MAB2223 (Merck KGaA, Darmstadt, Germany) diluted 1:5000 was added and incubated for 1 hour. Then, the membrane was washed twice with 1x TBS-T for 15 minutes each. The membrane was then incubated with the 926-32210 IRDye 800CW anti-mouse secondary antibody 1:5000 (LI-COR BioSciences). Then, the membrane was washed two times with 1x TBS-T for 15 minutes each time and scanned using in Li-COR's Odyssey Infrared Imaging System (LI-COR BioSciences).

3.8. 3-Nitrotyrosine Determination

For 3-NT measurement, the same procedure that was used in the determination of carbonyl groups was done. The nitrocellulose membrane was then incubated with 10% methanol, to its permeabilization, and washed in water and 1x TBS-T.

Then, the spermatozoa samples were initially diluted in 1x TBS to obtain a final protein concentration of 1 ηg/μL. After, 100 μL of these samples was slot-blotted into the nitrocellulose membrane.

After the above steps, 5% milk in 1x T-TBS was used to block the nitrocellulose membrane. Then, we incubated the membrane with primary antibody anti-nitrotyrosine, cat# 06-284, lot# DAM1514077, rabbit polyclonal (Merck KGaA, Darmstadt, Germany), diluted 1:1000. Then, it was washed two times with 1x T-TBS for 15 minutes each time. It was added the secondary antibody 926-68071 IRDye 680RD anti-rabbit, 1:5000 (LI-COR BioSciences), for one hour. The membrane was again washed for two times with 1x T-TBS for 15 minutes and, finally, it was revealed in Li-cor's Odyssey Infrared Imaging System (LI-COR BioSciences).

3.9. Immunocytochemistry of human sperm

An aliquot of fresh washed sperm was placed onto a glass coverslip and dried at RT, in a six-well plate containing one coverslip per well. To each well 4% paraformaldehyde in 1x PBS was gently added and left to stand for 10 minutes. Subsequently, sperm were washed twice with 1x PBS for 5 minutes. For permeabilization, methanol solution was added for 2 minutes and then the specimens were washed twice with 1x PBS for 5 minutes and blocked for 30 minutes with 3% BSA in 1x PBS, before incubation with primary antibodies (rabbit anti-GPx1, 1:1000, rabbit anti-GPx4, 1:250 and mouse anti-SOD1, 1:600) for 1 hour and 30 minutes at RT. After three washes with 1x PBS, the fluorescent-labeled secondary antibody goat anti-rabbit Texas-Red, 1:300 (MolecularProbes, Life Technologies, USA) and anti-mouse Alexa 488, 1:400 (MolecularProbes, Life Technologies, USA) was added and the coverslips incubated for 1 hour. Finally, three washes with 1x PBS were performed and coverslips were mounted on microscope glass slides with one drop of anti-fading reagent containing DAPI for nucleic acid staining (Vectashield, Vector Laboratories Burlingame, California, USA). Images were acquired using an Olympus IX81 epifluorescence microscope and digital camera, equipped with appropriate software (Olympus Portugal - Opto-Digital Tecnologias, S.A., Lisboa, Portugal).

3.10. Statistical Analysis

A longitudinal study approach was used to estimate the changes in sperm quality at three distinct time points. It was performed a descriptive statistic (statistical measures and bar graph) for each assay followed by box-plot and error bars 95% CI graph. To detect significant alterations for each parameter between weeks, we performed the ANOVA for repeated measures (Parametric test) or the Friedman Test (Non-parametric test). For each parameters with significant differences between the three weeks, we performed three bar graphs, where each graph represents the differences between weeks (TP3 - TP1, TP3 - TP2 and TP2 - TP1) as a function of the parameter evaluated for each of the 15 volunteers. Subsequently, to identify the differences between each TP, for each parameter were performed 3 paired tests. In the parametric case, it was used the t-Student test for paired samples and, in the nonparametric case, the Sign test or the Wilcoxon test (validated symmetry assumption) was used. Finally, in order to study the dependence between two variables, it was calculated the Pearson or Spearman correlation

coefficients with p-value (parametric or nonparametric methods, respectively). The use of parametric or non-parametric methods was judged by Shapiro-Wilk test for normality. All statistical analysis is realized with the support of IBM SPSS 20.0 statistical software and the fixed significance level of 5%.

4. Results

4.1. Lifestyle alteration

Semen samples of 15 volunteers were collected before (TP1) and after (TP2 and TP3) the academic festivities. First, the basic semen analysis of all samples were performed (data in **Appendix 8.3**) and, subsequently, the samples were processed to evaluate the accessory glands function (data in **Appendix 8.4**) and to perform the TAS, SOD1 and GPx4 expression (data in **Appendix 8.5**), and carbonyl and 3-NT groups detection (data in **Appendix 8.6**). Immunocytochemistry analysis was also performed with fresh human sperm cells.

The answers of the questionnaires of the 15 volunteers in the 3 TPs were evaluated to assess the alterations in the consumption of alcohol and nicotine.

4.1.1. Alcohol consumption

Initially, consumption of alcohol during the 3 TPs was evaluated. A descriptive statistics of the answers of the volunteers was obtained and is described in **Table 7**. The results were calculated considering that each drink has a reference value of alcohol grams: a beer has approximately 15,84 g of alcohol, a wine glass has 16,8 g, a white alcoholic drink has 80 g and a shot has 16 g.

Table 7 – Descriptive statistics of the alcohol consumption. The results are presented in grams.

	Alcohol consumption		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	17,49	173,23	18,94
Std. Deviation	45,07	150,67	24,83
Minimum	,00	,00	,00
Maximum	179,20	480,00	80,00

TP: time point

Analyzing **Table 7**, it is possible to observe that during TP2 an increase of approximately ten times greater in alcohol consumption occurs when compared to TP1. To visualize this alteration, a box plot was performed (**Figure 9**).

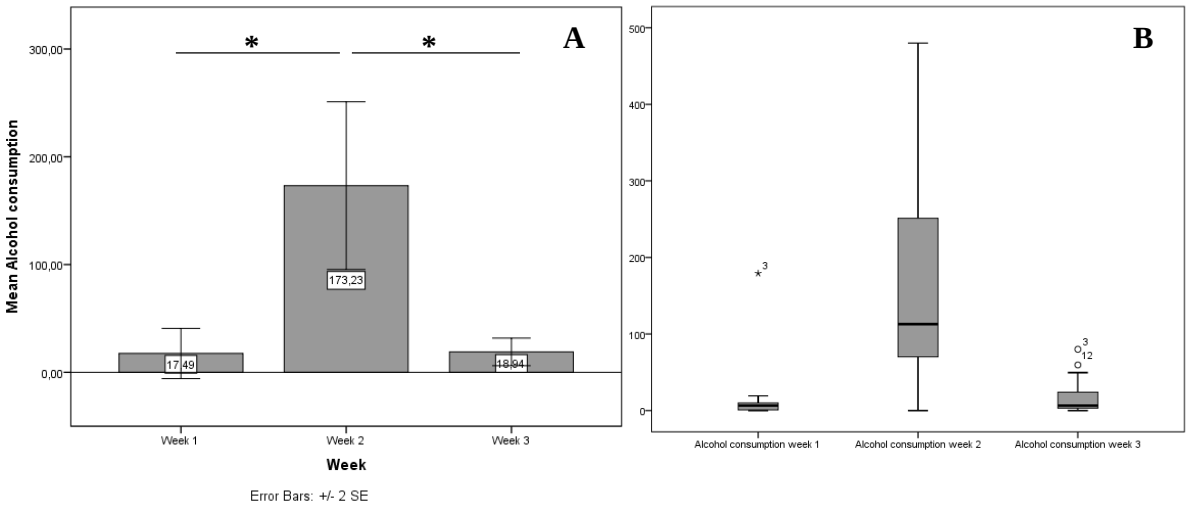

Figure 9 - (A) Bar graph and error bars of alcohol consumption; **(B)** Box plot and outliers of alcohol consumption at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

To determine if the differences between the three weeks were statistically significant, a Friedman test was performed and a statistically significant difference was found (p=0,00).

To confirm it, a Sign Test was performed. The differences between TP1 and TP3 were not statistical significant (p=0,267). However, as expected, the differences between TP1 and TP2 were statistically significant (p=0,00), as well as between TP2 and TP3 (p=0,00).

The sorted differences between TP1/TP2, TP1/TP3 and TP2/TP3 as a function of the samples may be consulted in **Appendix 8.7.1.1**.

4.1.2. Nicotine consumption

After the alcohol consumption, we focused in the consumption of tobacco, more concretely the consumption of nicotine. To evaluate this alteration, we first obtained a descriptive statistics analysis for the answers of the questionnaires, considering that each cigarette has 1,2 mg of nicotine. The results are presented in **Table 8**.

Table 8 – Descriptive statistics of the nicotine consumption. The results are presented in milligrams.

	Nicotine consumption		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	,9173	1,920	1,5207
Std. Deviation	2,08825	2,8252	3,60453
Minimum	,00	,0	,00
Maximum	6,00	7,2	12,00

TP: time point

Analyzing **Table 8**, it is possible to observe that nicotine consumption during TP2 increased twice relatively to TP1. At TP3, the nicotine consumption decreased. To visualize these alterations, a bar graph and a box plot was performed (**Figure 10**).

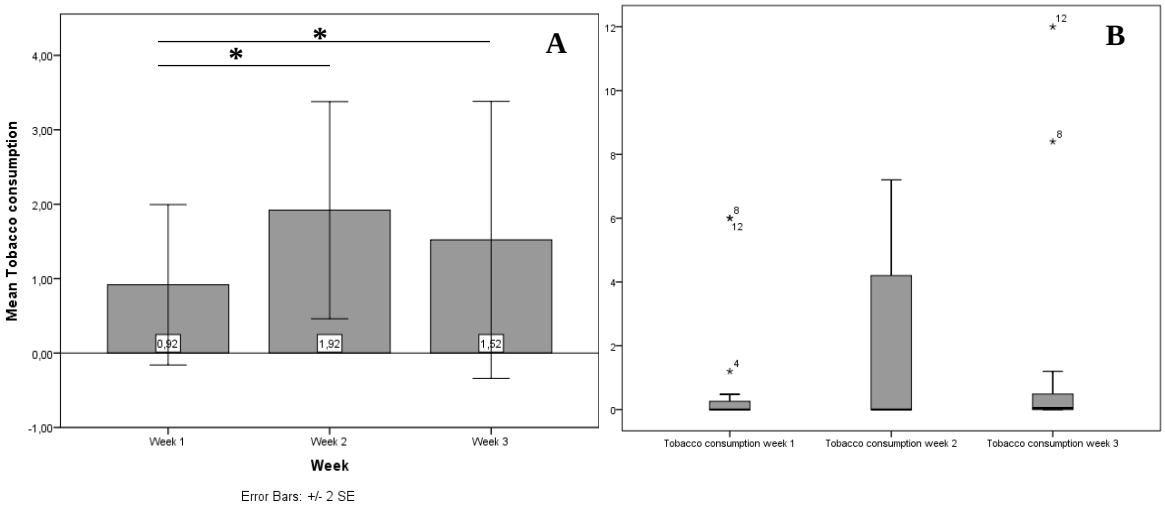

Figure 10 - (A) Bar graph and error bars of alcohol consumption; **(B)** Box plot and outliers of nicotine consumption at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

Subsequently, a Friedman test was performed, being observed a statistically significant difference between the three weeks (p=0,043).

At last, a Sign Test was performed. The increase of the nicotine consumption of TP1 to TP2 was statistically significant (p=0,0315), as well as from TP1 to TP3 (p=0,035). However, the differences between TP2 and TP3 were not statistical significant (p=1,000).

The sorted differences between TP1/TP2, TP1/TP3 and TP2/TP3 as a function of the samples are shown in **Appendix 8.7.1.2**.

4.2. Impact of lifestyle changes on:

4.2.1. Basic semen parameters

Basic semen analysis (data in **Appendix 8.3**) was firstly evaluated to understand the impact of lifestyle alterations on semen quality. All the data were submitted to a statistical analysis. The results with statistical significance will be presented in this section. The remaining data can be found in the **Appendix 8.7.2**, together with a descriptive statistics and the test performed to determine if the differences between the three weeks studied were statistically significant.

4.2.1.1. Semen volume

The semen volume, which is composed by seminal vesicles and prostate gland secretions, was assessed. To evaluate the possible alterations, we obtained the descriptive statistics analysis of the 15 volunteers, which is described in **Table 9**.

Table 9 – Descriptive statistics of the semen volume. The results are expressed in milliliters.

	Semen volume		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	3,2	3,4	2,5
Std. Deviation	1,3	2,0	1,6
Minimum	1,0	,7	1,0
Maximum	6,5	7,0	6,0

TP: time point

By observing **Table 9**, we can conclude that the mean of semen volume has slightly decreased in the TP3, compared to TP1 and TP2. A bar graph and error bars of semen volume was performed (**Figure 11 A**). A box-plot of the samples to visualize the median and the distribution of the samples and the presence of outliers was also performed (**Figure 11 B**).

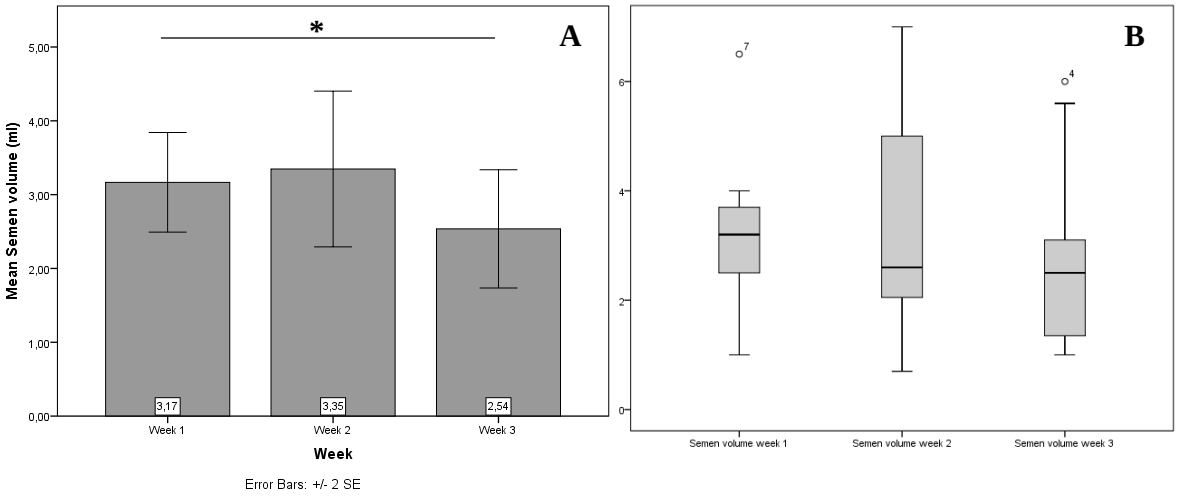

Figure 11 - (A) Bar graph and error bars of semen volume; **(B)** Box plot and outliers of semen volume at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

A Friedman test, a non-parametric test that compares two or more paired samples, was performed. We can conclude that the samples are significantly different between the different TPs, since the p value is < 0,05 (p= 0,042).

To confirm if there was a significant difference in the semen volume between the time points, it was performed a t-Student Test for paired samples and a Sign Test.

By performing the t-Student Test, we concluded that there was not a significant difference in the semen volume between TP1 and TP2, because of the p value > 0,05 (p= 0,635). In the same way, there was not a significant difference in the semen volume between TP2 and TP3 (p=0,302). However, TP1 and TP3 showed a p value < 0,05 (p=0,006), which means that the decrease of the semen volume from TP1 to TP3 was statistically significant.

The sorted differences between TP1/TP2, TP1/TP3 and TP2/TP3 as a function of the samples may be consulted in **Appendix 8.7.2.2**.

4.2.1.2. Progressive motility

To understand if lifestyle alterations had a negative impact on sperm progressive motility, a descriptive statistics analysis was obtained (**Table 10**).

Table 10 - Descriptive statistics of the semen volume. The results are expressed in percentage.

	Progressive motility		
	TP1	**TP2**	**TP3**
N	14	15	15
Mean	42,1	26,7	29,5
Std. Deviation	15,9	14,0	18,2
Minimum	6	3	3
Maximum	62	59	60

TP: time point

Analyzing **Table 10**, the mean percentage of spermatozoa with progressive motility at TP1 was approximately 42,1%, while at TP2 the mean percentage decreased to 26,7%. In the same way, although the percentage of spermatozoa has slightly increased in relation to TP2, the mean value of spermatozoa with progressive motility at TP3 remained below to the basal levels (TP1). A bar graph and error bars of progressive motility was performed (**Figure**

12 A). A box-plot of the samples to visualize the median and the distribution of the samples and the presence of outliers was also performed (**Figure 12 B**).

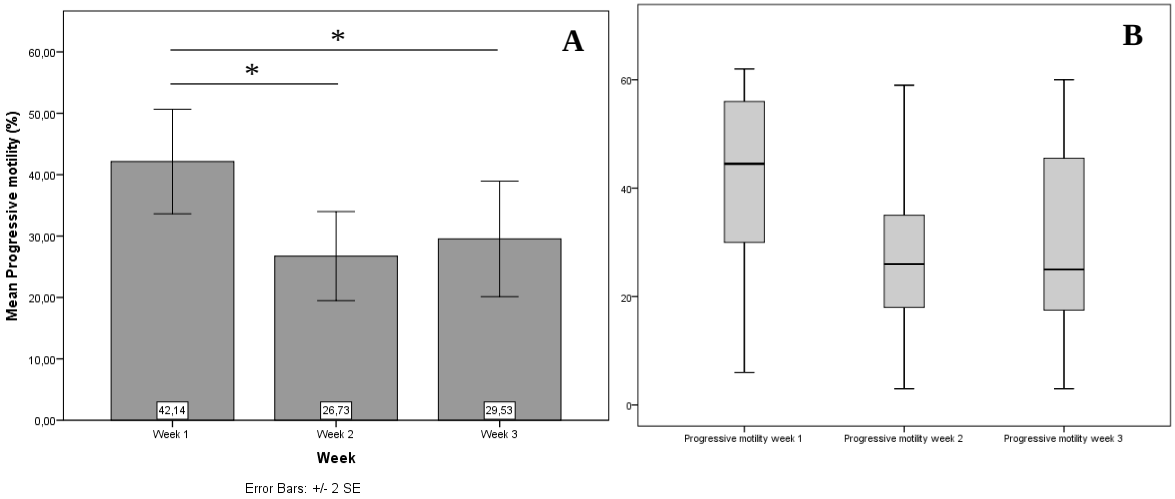

Figure 12 – **(A)** Bar graph and error bars of progressive motility; **(B)** Box plot and outliers of progressive motility at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

An ANOVA with repeated measures, a parametric test used to compare three or more paired samples, was performed to determine if the differences between the three weeks were statistically significant. For the progressive motility, we can conclude that the samples are significantly different between the different TPs (p value= 0,005).

We performed a t-Student test for paired samples to confirm if the differences between the three time points were statistically significant. By performing the t-Student Test, we concluded that there was a significant difference in the progressive motility between TP1 and TP2 (p=0,004), as well as between TP1 and TP3 (p=0,004). However, the difference between TP2 and TP3 was not statistical significant (p=0,475).

The sorted differences between TP1/TP2, TP1/TP3 and TP3/TP2 as a function of the samples may be consulted in **Appendix 8.7.2.5**.

4.2.1.3. Non progressive motility

To evaluate the impact of lifestyle changes on seminal function, non progressive motility was also evaluated. A descriptive statistics of the 15 volunteers was obtained, which is described in **Table 11**.

Table 11 – Descriptive statistics of the non progressive motility. The results are presented in percentage of spermatozoa.

	Non progressive motility		
	TP1	**TP2**	**TP3**
N	14	15	15
Mean	11,3	20,5	19,6
Std. Deviation	4,7	9,6	7,9
Minimum	5	9	11
Maximum	21	37	38

TP: time point

Analyzing **Table 11**, the mean percentage of spermatozoa with non progressive motility increased approximately 2 times between TP1 and TP2. In the same way, although the percentage of spermatozoa has slightly decreased in relation to TP2, the mean value of spermatozoa with non progressive motility at TP3 remained above to the basal levels (TP1).

A bar graph and error bars of progressive motility was performed (**Figure 13 A**). A box-plot of the samples to visualize the median and the distribution of the samples and the presence of outliers was also performed (**Figure 13 B**).

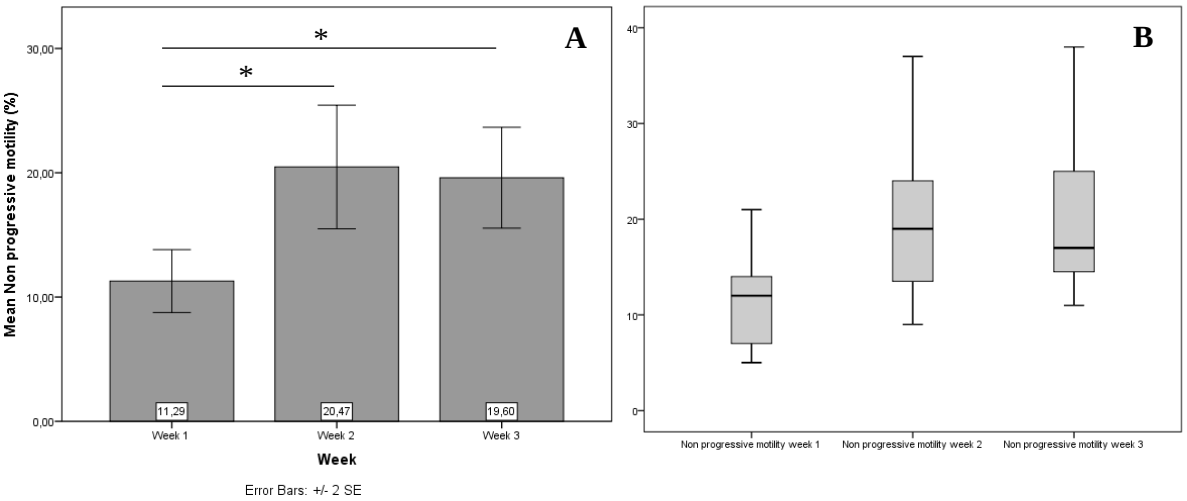

Figure 13 – **(A)** Bar graph and error bars of progressive motility; **(B)** Box plot and outliers of non progressive motility at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

A Friedman test was performed and we can conclude that the samples are significantly different between the different TPs, since the p value is < 0,05 (p= 0,046).

To confirm if there was a significant difference in the non-progressive motility between the time points, it was performed a Wilcoxon Test and a t-Student Test for paired samples. By performing the Wilcoxon test, we concluded that there was a significant difference in the progressive motility between TP1 and TP2 (p=0,013), as well as between TP1 and TP3 (p=0,007), performed by the t-Student test. In the other hand, the differences between TP2 and TP3 were not statistical significant (p=0,888).

The sorted differences between TP1/TP2, TP1/TP3 and TP2/TP3 as a function of the samples may be consulted in **Appendix 8.7.2.6.**

4.2.1.4. Normal morphological sperm

In **Table 12** are described a descriptive statistics of the mean percentage of normal morphological sperm to understand if the lifestyle alterations had a negative impact in this parameter.

Table 12 – Descriptive statistics of the normal sperm. The results are presented in percentage of spermatozoa.

	Normal sperm		
	TP1	**TP2**	**TP3**
N	14	14	13
Mean	11,57	9,71	9,38
Std. Deviation	5,958	5,810	4,610
Minimum	3	2	3
Maximum	19	22	18

TP: time point

Analyzing **Table 12**, the mean percentage of normal spermatozoa at TP1 was approximately 11,57%, while at TP2 and TP3 the mean percentage decreased to 9,71% and 9,38% respectively.

A bar graph and error bars of normal sperm was performed (**Figure 14 A**). A box-plot of the samples was also performed to visualize the median and the distribution of the samples and the presence of outliers (**Figure 14 B**).

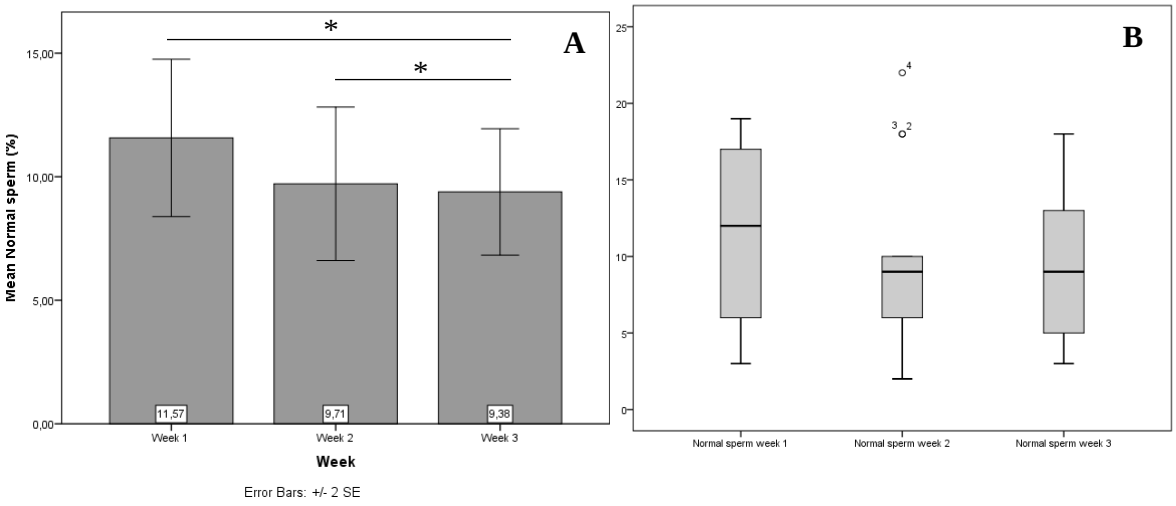

Figure 14 - (A) Bar graph and error bars of normal sperm; **(B)** Box plot and outliers of normal sperm at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

An ANOVA with repeated measures was performed, in which we can conclude that the samples are significantly different between the different TPs (p= 0,044).

To confirm the statistical significance, a t-Student Test for paired samples was performed. The differences between TP1 and TP2 were not statistical significant (p=0,144), as well as between TP2 to TP3 (p=0,786). However, the decrease of the normal sperm forms from TP1 to TP3 was statistically significant (p=0,018).

The sorted differences between TP1/TP2, TP1/TP3 and TP3/TP2 as a function of the samples may be consulted in **Appendix 8.7.2.8**.

4.2.1.5. Tail defects

To understand if lifestyle alterations had a negative impact on sperm tail defects, a descriptive statistics analysis was obtained (**Table 13**).

Table 13 – Descriptive statistics of the tail defects. The results are represented in percentage of spermatozoa.

	Tail defects		
	TP1	**TP2**	**TP3**
N	14	14	13
Mean	27,50	36,57	34,46
Std. Deviation	10,376	10,740	11,709
Minimum	12	20	19
Maximum	43	60	51

TP: time point

Analyzing **Table 13**, the mean percentage of spermatozoa with tail defects at TP1 was approximately 27,5%, while at TP2 the mean percentage increased to 36,57%. Similarly, although the percentage of spermatozoa has slightly decreased in relation to TP2, the mean value of spermatozoa with tail defects at TP3 is higher in relation to the basal levels (TP1).

A bar graph and error bars of tail defects was performed (**Figure 15 A**). A box-plot of the samples was performed to visualize the median and the distribution of the samples and the presence of outliers (**Figure 15 B**). When analyzing the graphs of the three TPs, it was possible to verify, once again, that there was an increase of tail defects at TP2 and TP3 in relation to TP1.

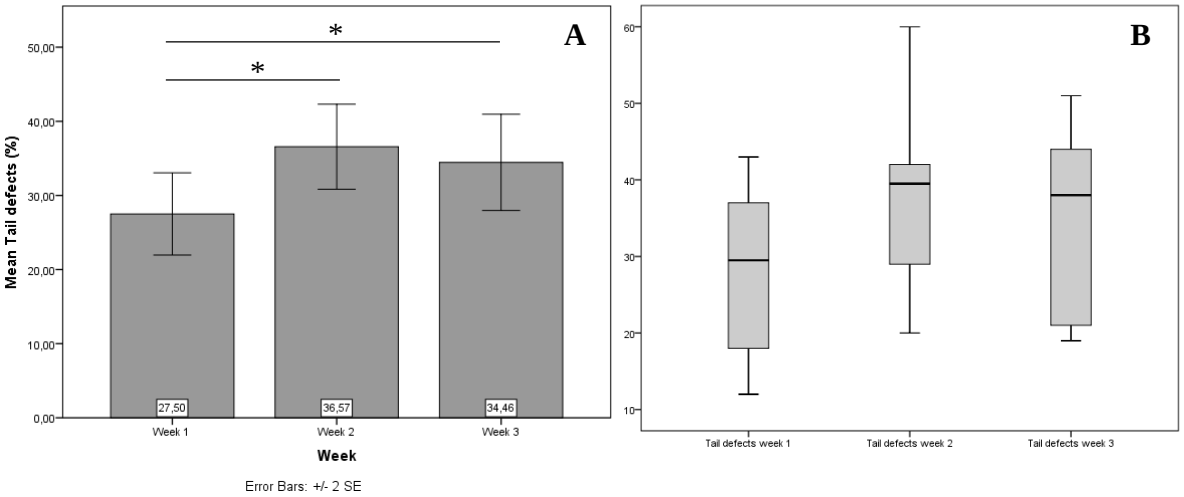

Figure 15 – **(A)** Bar graph and error bars of tail defects; **(B)** Box plot and outliers of tail defects at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

An ANOVA with repeated measures was performed, showing that the samples are significantly different between the different TPs (p= 0,005).

To confirm if there was a significant difference in the tail defects between the time points, it was performed a t-Student Test for paired samples. By performing the T-Student Test, a significant difference in the tail defects between TP1 and TP2 (p=0,002), as well as between TP1 and TP3 (p=0,002) was observed. The differences between TP3 and TP2 were not statistical significant (p=0,386).

The sorted differences between TP1/TP2, TP1/TP3 and TP3/TP2 as a function of the samples may be consulted in **Appendix 8.7.2.11**.

4.2.2. Accessory glands function

After performing basic semen analysis, accessory glands function of all volunteers (data in **Appendix 8.4**) was subsequently assessed to evaluate the influence of lifestyle alterations during academic festivities in these glands function. All the data were submitted to a statistical analysis. The results with statistical significance will be presented in this section. The remaining data can be found in the **Appendix 8.7.3**.

4.2.2.1. Total concentration of neutral α-glucosidase per ejaculate

In **Table 14**, a descriptive statistics analysis relative to total concentration of NAG in ejaculate is represented to understand the impact of lifestyle alterations on this parameter.

Table 14 - Descriptive statistics of the total NAG. The results are represented in mIU per ejaculate.

	Total NAG concentration		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	47,80	46,17	29,86
Std. Deviation	44,70	37,68	28,87
Minimum	,00	5,97	,00
Maximum	190,42	138,46	122,74

TP: time point

Analyzing **Table 14**, the mean total concentration of NAG at TP1 (47,8 mIU) and TP2 (46,17 mIU) remained practically unchanged. However, at TP3, the mean concentration decreased to 29,86 mIU.

A bar graph and error bars of total NAG concentration was performed (**Figure 16 A**). A box-plot of the samples was also performed to visualize the median and the distribution of the samples and the presence of outliers (**Figure 16 B**). When analyzing the graphs, it is possible to confirm, once again, that there was a visible decrease of total concentration of NAG at TP3.

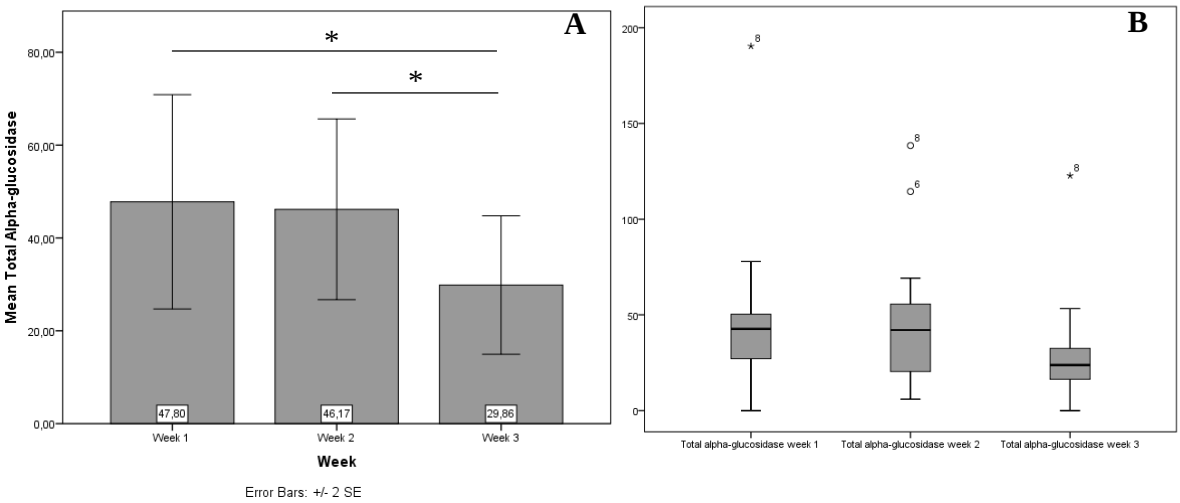

Figure 16 - (A) Bar graph and error bars of total NAG concentration; **(B)** Box plot and outliers of total NAG concentration at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

We performed a Friedman test for the total concentration NAG and we can conclude that the samples are significantly different between the TPs, since the p value is < 0,05 (p= 0,006). To confirm if there was a significant difference in the total neutral α-glucosidase between the time points, a Sign Test was performed, in which we observed that there was a significant difference in the total concentration of neutral α-glucosidase per ejaculate between TP1 and TP3 (p=0,035), as well as between TP2 and TP3 (p=0,007). The differences between TP1 and TP2 were not statistical significant (p=0,607).

4.2.3. Antioxidant defense system

To understand the implication of lifestyle alterations in the oxidative balance of the sperm cells, the total antioxidant status, the expression of two important antioxidant enzymes and OS-induced protein damage were also evaluated (data in **Appendix 8.5** and **8.6**). All the data were submitted to a statistical analysis. The results with statistical significance will be presented in this section and the remaining data can be found in the **Appendix 8.7.4**.

4.2.3.1. Total antioxidant status (TAS)

The TAS assay, performed to evaluate the antioxidant capacity of the sperm cells, was also assessed. As observed in **Table 15**, the mean values increased along the TPs studied.

Table 15 – Descriptive statistics of the total antioxidant status. The results are presented in mmol/l.

	TAS		
	TP1	**TP2**	**TP3**
N	14	14	14
Mean	1,43	1,62	1,97
Std. Deviation	,91	,79	,75
Minimum	,27	,37	,07
Maximum	2,65	2,62	3,14

TP: time point; **TAS**: total antioxidant status

To visualize the previous results, it was performed a bar graph and box plot, which are represented in **Figure 17**.

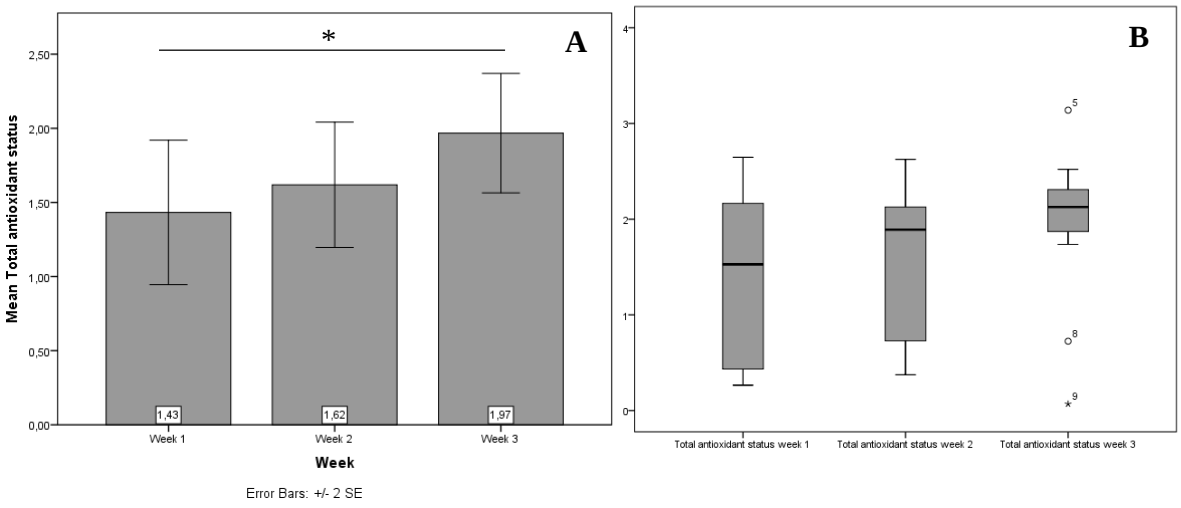

Figure 17 - **(A)** Bar graph and error bars of TAS; **(B)** Box plot and outliers of TAS at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

A Friedman test was performed, and a statistically significant difference between the three weeks was observed (p=0,030).

To confirm it, a t-Student Test for paired samples and a Sign Test was used. The differences between TP1 and TP2, as well as between TP2 and TP3, were not statistical significant (p=0,289 and p=0,09, respectively). However, the increase of the total antioxidant status from TP1 to TP3 was statistically significant (p=0,0254).

The sorted differences between TP1/TP2, TP1/TP3 and TP3/TP2 as a function of the samples may be consulted in **Appendix 8.7.4.1.**

4.2.3.2. Antioxidant enzymes immunolocalization

Since in human sperm cells, only SOD1 immunolocalization was previously evaluated, we analyzed the subcellular localization of antioxidant enzymes SOD1, GPx1 and GPx4 in mature human ejaculated sperm. Two human sperm samples and a total of 100 cells were evaluated. As seen in **Figure 18 A**, SOD1 immunoreactivity was predominantly detected in the head (100%), midpiece (95,54%) and in the entire length of the flagellum (90,18%). GPx1 immunoreactivity (**Figure 18 D**) was predominantly detected in the midpiece (98,08%), but also in the head (97,12%), and tail (91,35%). Additionally, spermatozoa with no labelling represented approximately 1,92% of the sample. In relation to GPx4 immunolocalization, 100% of sperm cells showed immunoreactivity in the head, but in 96% of spermatozoa the staining also appeared in the tail region. However, immunoreactivity is more intense in midpiece region (96%) (**Figure 18 G**). We also observed some fluorescence in auto-fluorescence and negative control samples.

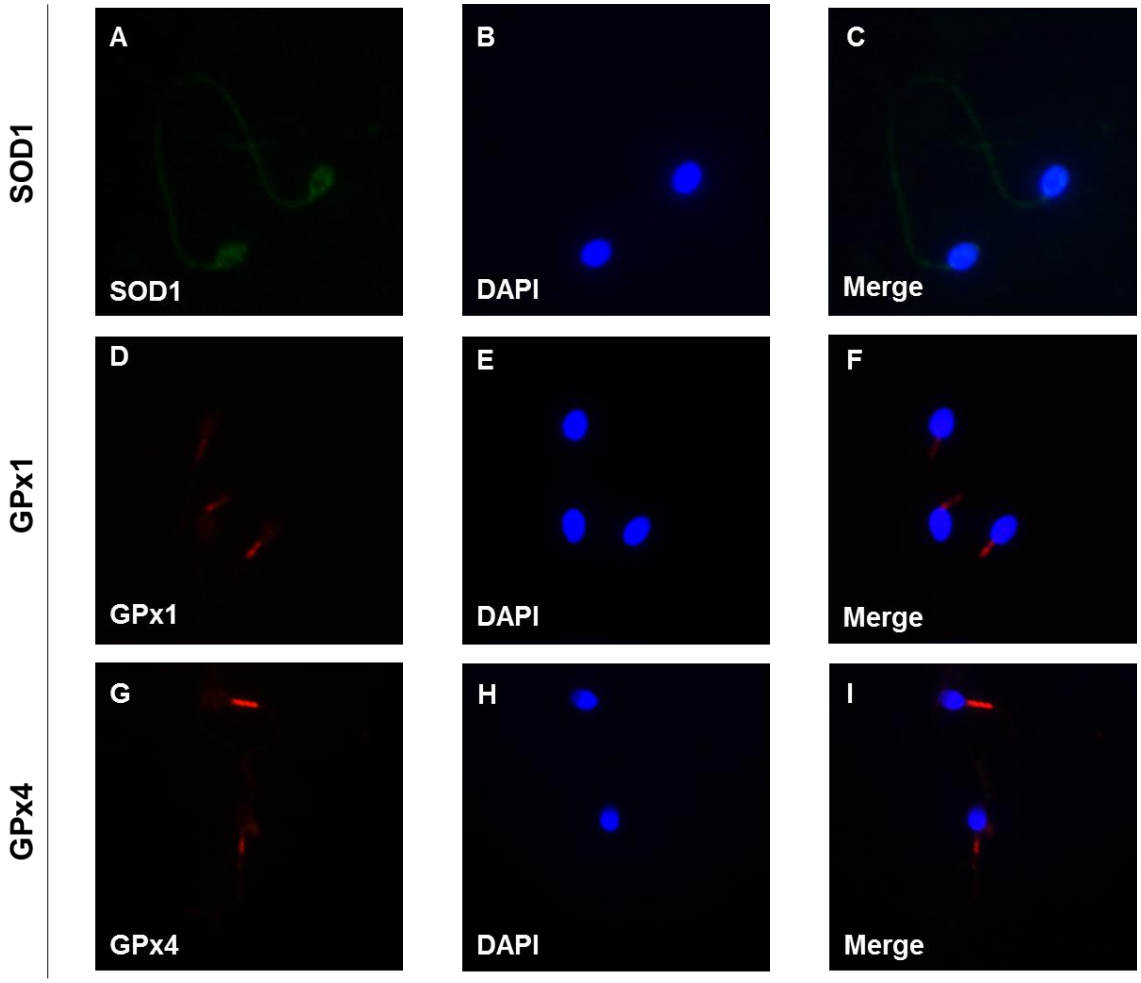

Figure 18 - Immunolocalization of SOD1, GPx1 and GPx4 enzymes on human spermatozoa. DAPI is used as the counterstain for nucleus. Magnification 63x.

Taking account the immunolocalization, above described, and the biological role of the enzymes analyzed, it was evaluated the expression of SOD1 and GPx4.

4.2.3.3. SOD1 expression

Subsequently, we measured the expression of the antioxidant enzyme SOD1 (**Figure 19**) by Western Blot in a total of 14 samples. To standardize the samples, it was used the amount of β-tubulin, performing a ratio SOD1/β-tubulin, for each sample.

The data of SOD1 expression was submitted to a statistical analysis and no statistical differences between the TPs were found (the data are found in the **Appendix 8.7.4.2)**.

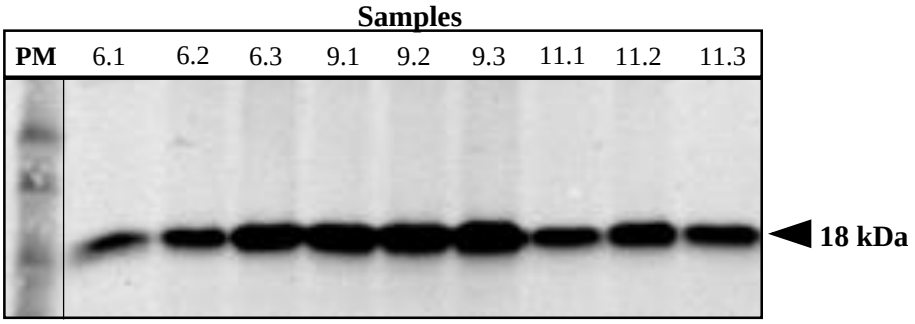

Figure 19 - Representative blot of SOD1 expression detected by Western blot. **PM:** protein marker.

4.2.3.4. GPx4 expression

The expression of the antioxidant enzyme GPx4 (**Figure 20**) was also assessed by Western Blot in a total of 14 samples and standardized with the amount of β-tubulin, performing a ratio GPx4/β-tubulin, for each sample.

The data of GPx4 expression was submitted to a statistical analysis and no statistical differences between the TPs were found (the data are found in **the Appendix 8.7.4.3**).

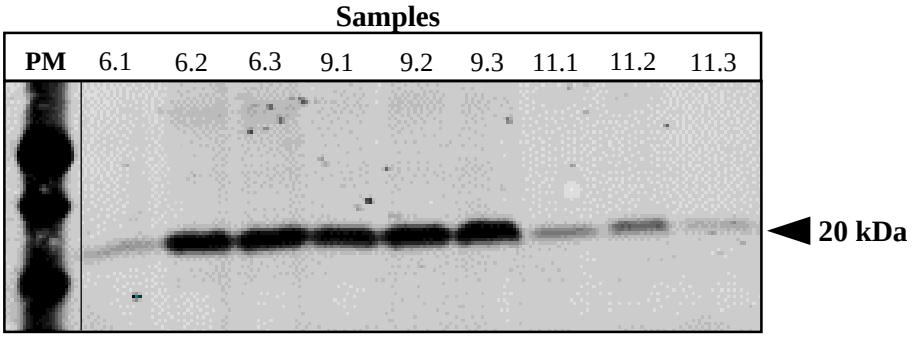

Figure 20 - Representative blot of GPx4 expression detected by Western blot. **PM:** protein marker.

4.2.4. Protein oxidation

All the data were submitted to a statistical analysis and the results with statistical significance will be presented in this section.

4.2.4.1. Carbonyl groups

The presence of carbonyl groups in the samples was assessed by the slot-blot technique (**Figure 21**) and the results were standardized through the protein concentration used in this assay.

The data of carbonyl groups detection in the three TPs was submitted to a statistical analysis and no statistical differences between the TPs were found (the data are presented in the **Appendix 8.7.5.1**).

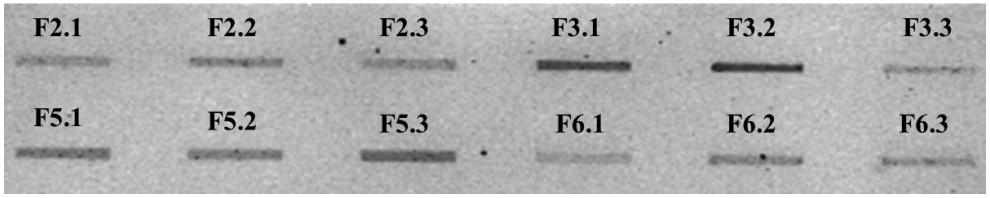

Figure 21 - Results obtained with CG determination by slot-blot.

4.2.4.2. 3-Nitrotyrosine groups

Subsequently, the determination of 3-NT in the samples was assessed by slot-blot technique (**Figure 22**). The results were standardized through the protein concentration used in this assay.

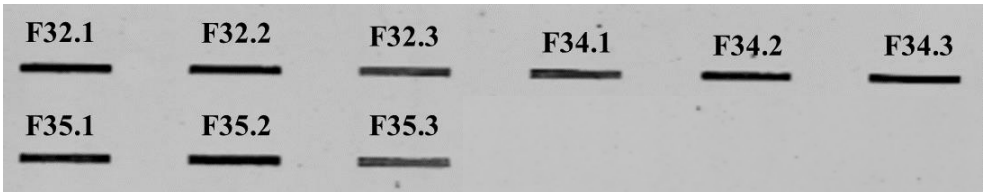

Figure 22 - Results obtained with 3-NT determination by slot-blot.

Then, to understand if lifestyle alterations lead to an increase in 3-NT levels, a descriptive statistics analysis was obtained and is described in **Table 16**.

Table 16 – Descriptive statistics of the 3-nitrotyrosine presence.

	3-NT		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	201,21	261,69	194,49
Std. Deviation	109,24	107,49	76,84
Minimum	69,08	56,11	66,24
Maximum	383,35	419,37	333,03

TP: time point; **3-NT**: 3-nitrotyrosine

A box plot, which is represented in **Figure 23**, was also performed. It is possible to see that during TP2 occurred an increase in this protein modification parameter.

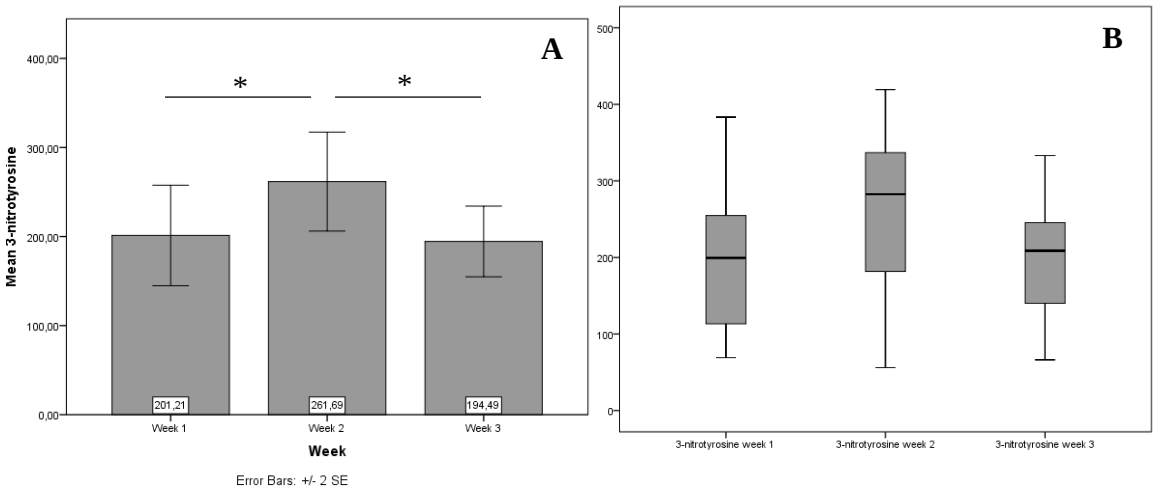

Figure 23 - (A) Bar graph and error bars of 3-nitrotyrosine; **(B)** Box plot and outliers of 3-nitrotyrosine at the three weeks. Horizontal lines with an asterisk indicate significant differences between groups.

To determine if the differences between the three weeks were statistically significant, an ANOVA with repeated measures was performed and a statistically significant difference between the three weeks was observed (p=0,025).

To confirm it, a t-Student Tests for paired samples was performed. The differences between TP1 and TP3 were not statistical significant (p=0,789). The increase of the 3-nitrotyrosine levels from TP1 to TP2 were statistically significant (p=0,013), as well as between TP2 and TP3 (p=0,016).

The sorted differences between TP1/TP2, TP1/TP3 and TP3/TP2 as a function of the samples may be consulted in **Appendix 8.7.5.2.**

4.3. Relation between seminal quality and oxidative stress parameters

After performing and evaluating the basic semen analysis parameters, accessory gland function, TAS assay, Western Blot (SOD and GPx4 expression levels) and Slot-blot (3-NT and CG levels), we statistically evaluated the data to detect the possible correlations between the basic semen parameters and accessory gland function parameters with the alterations in

antioxidants and oxidized proteins levels. For the correlations analysis, the three TPs were merged and, therefore, in this analysis will be used a total of 45 semen samples. The correlations were evaluated, performing a Spearman or a Pearson correlation coefficient with p-value. The results with statistical significance will be presented in this section, while the remaining data can be found in the **Appendix 8.7.6.**

Observing **Table 17**, a moderate positive linear relationship was observed between midpiece defects and 3-NT (p=0,049), as well as between the age of volunteers and 3-NT (p=0,029) and semen volume and carbonyl groups (p=0,022). Furthermore, a negative linear relationship was found between sperm concentration and TAS (p=0,03) and SOD1 expression (p=0,019).

A positive linear was also observed between sperm imotility and SOD1 expression (p=0,033). Additionally, a negative correlation between progressive motility and carbonyl groups (p=0,047), performed by Spearman's correlation, was observed.

The relationship between basic semen analysis parameters and accessory gland function parameters was also evaluated. A moderate negative linear relationship between sperm head defects and citric acid concentration (p=0,01) was found.

A moderate negative linear relationship between age and NAG concentration (p=0,043) and between semen volume and NAG concentration (p=0,016) was found. A moderate relationship between the next pairs was found: sexual abstinence with citric acid concentration (p=0,029); semen volume with total concentration of fructose (p=0,001) and NAG (p=0,012); sperm concentration with citric acid (p=0,002) and NAG (p=0,029) concentration and with total concentration of NAG (p=0,028); total number of spermatozoa per ejaculate with total concentration of citric acid (p=0,001), fructose (p=0,002) and NAG (p=0,001). Furthermore, semen volume also positively correlates with total concentration of citric acid (p=0,000). Normal sperm forms positively correlates with total concentration of NAG (p=0,043).

A moderate positive linear correlation was obtained between TAS and SOD1 (p=0,028) and GPx4 (p=0,028). A strong positive linear relationship between SOD1 and GPx4 was found (p=0,000).

The relationship between accessory gland function and oxidative stress parameters was also assessed. A moderate positive linear correlation between GPx4 expression and total

fructose concentration (p=0,03) was obtained. However, a moderate negative linear relationship was verified between GPx4 and citric acid concentration (p=0,021).

A moderate negative linear relationship was found between the next pairs: TAS and fructose (p=0,014) and NAG concentration (p=0,017); 3-NT and NAG concentration (p=0,023).

Lastly, the relationship between alcohol and nicotine consumption and stress oxidative parameters was also studied. A moderate positive linear relationship between 3-NT and alcohol consumption (p=0,014) was found. In the other hand, a moderate negative linear relationship between alcohol consumption and fructose concentration (p=0,009) and total concentration of fructose in seminal plasma (p=0,026) was also found.

Table 17 - Pearson (r) and Spearman (r_s) correlation coefficients between basic semen parameters, accessory glands function and oxidative stress parameters evaluated. * Correlation is significant at the 0.51 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed).

	Citric acid [] (mg/mL)	**Total citric acid (mg)**	**Fructose [] (mg/mL)**	**Total fructose (mg)**	**NAG [] (mg/mL)**	**Total NAG (mg)**	**TAS (mmol/L)**	**SOD1**	**GPx4**	**CG**	**3-NT**
Age (years)					r_s=-0,31* N=43						r_s=0,326* N=45
Sexual abstinence (days)	r_s=0,329* N=44										
Volume (mL)		r_s=0,515** N=45		r_s=0,493** N=43	r_s=-0,366* N=43	r_s=0,371* N=45			r_s=0,352* N=42	r_s=0,349* N=42	
Sperm [ ] (x10^6 spz/mL)	r_s=0,45* N=44				r_s=0,333* N=43	r_s=0,328* N=45	r_s=-0,335* N=42	r_s=-0,36* N=42			
Total sperm [ ] (x10^6 spz)		r_s=0,481** N=45		r_s=0,461** N=43		r_s=0,488** N=45					
Progressive motility (%)										r_s=-0,312* N=41	
Immotility (%)										r_s=0,334* N=41	
Normal sperm (%)						r_s=0,322* N=40					

Head defects (%)	r=-0,399** N=41						
Midpiece defects (%)							r=0,309 N=41
TAS (mmol/L)		r_s=-0,387* N=40	r_s=-0,377* N=40		r_s=0,339* N=42	r_s=0,34* N=42	
SOD1 expression				r_s=0,339* N=42		r_s=0,677** N=42	
GPx4 expression	r_s=-0,36* N=41		r_s=0,343* N=40	r_s=0,34* N=42	r_s=0,677** N=42		
3-NT			r_s=-0,347* N=43				
Alcohol consumption (g)		r_s=-0,392** N=43	r_s=-0,339* N=43				r_s=0,365* N=45

NAG: neutral α-glucosidase; **TAS**: total antioxidant status; **SOD1**: superoxide dismutase 1; **GPx4**: glutathione peroxidase 4; **CG**: carbonyl groups; **3-NT**: 3-nitrotyrosine; **spz**: spermatozoa

5. Discussion

The role of RS in human infertility has been investigated due to their toxic effects on spermatozoa biomolecules, specially lipids, proteins and nucleic acids, which consequently leads to the loss of important functions (Agarwal & Saleh 2002; Abdallah et al. 2009). Since the excessive consumption of substances of abuse, such as alcohol and tobacco smoking, are known to incite OS, leading to the impairment of cells function (Emanuele & Emanuele 2001; Taha et al. 2012), it is important to understand how the production and maturation of spermatozoa is affected and the subsequent implications for the function of these cells.

The impact of acute lifestyle changes during the academic festivities week, such as the consumption of alcohol, tobacco and drugs, in the male fertility was evaluated in "*Para o Frasco*" study. More specifically, it was evaluated the total antioxidant capacity of the sperm cells, the expression of specific antioxidant enzymes (GPx4 and SOD1) and the formation of some protein modifications, such as carbonyl and 3-NT groups. For this purpose, samples of 15 young male volunteers, in reproductive age, were collected at three moments: the first moment (TP1) was before the academic week, the second one (TP2) was one week after and the third moment (TP3) was around three months after this festivity. Since human spermatozoa takes 7 to 10 day to cross the epidydimis (Rowley et al. 1970), in TP2, we evaluated the effect of lifestyle changes on fully formed spermatozoa that were undergoing maturation in epididymis. In the other hand, in TP3, about 3 months after academic festivities, that is, equivalent to a spermatogenesis cycle, the impact of lifestyle alterations on the production of sperm cells at the testicular level was assessed.

Many lifestyle and behavioral factors can deteriorate semen quality, such as alcohol and tobacco consumption, caffeine and alterations in circadian rhythm (Kumar et al. 2009). However, in this study, since we cannot quantify accurately all these factors, only alcohol and nicotine consumption was assessed, considering a reference value for each drink and cigarette. The analysis of the questionnaires at each TP revealed an increase in alcohol consumption between TP1 and TP2, as well as, in nicotine consumption. In TP3, both consumptions decreased.

To understand the impact of the abusive consumption of these substances on semen quality, basic semen parameters, accessory gland function and OS parameters were assessed. In **Table 18** the studied parameters with statistical significance are represented. At TP2, a significant decrease in the percentage of spermatozoa with progressive motility, as well as, an increase in non progressive motility and tail defects were observed. At TP3, the progressive

motility also decreased, together with a diminution in semen volume and tin he percentage of spermatozoa with normal morphology. On the other hand, non progressive motility and tail defects suffered an increase relatively to basal levels (TP1). These results are in agreement with previous studies (Ferreira et al. 2012; Condorelli et al. 2014; Hamad et al. 2014; Jensen, Gottschau, et al. 2014).

Table 18 – Overview of the studied parameters with statistical differences between the TPs.

	TP1 to TP2	TP1 to TP3	TP2 to TP3
Semen Volume		↓	
Progressive motility	↓	↓	
Non progressive motility	↑	↑	
Normal sperm		↓	
Tail defects	↑	↑	
Total NAG [ ]		↓	↓
TAS		↑	
3-NT	↑		↓

TP: time point; **NAG:** neutral α-glucosidase; **TAS**: total antioxidant status; **3-NT**: nitrotyrosine

Progressive motility is an important seminal parameter to evaluate sperm function, since only progressive spermatozoa are capable of crossing cervical mucus and penetrating the oocyte (Ohl & Menge 1996). The detection of many components of tobacco smoke in seminal plasma of smokers, such as nicotine, cotinine (the major metabolite of nicotine), cadmium (Cd), and others, suggests that these compounds cross the BTB and create an adverse environment for the production of spermatozoa (Sepaniak et al. 2006; Chohan & Badawy 2010). Furthermore, Cd has the ability to mimic other divalent cations, such as calcium (Ca^{2+}) (Cheng & Mruk 2012). PMCA4 is a plasma membrane Ca^{2+}-ATPase, located at the principal piece of sperm tail, where Ca^{2+} flux is higher. It was demonstrated that PMCA4 null mice were infertile due to a loss in motility (Tempel & Shilling 2007). Since Cd may mimic Ca^{2+}, the replacement of this ion in the plasma membrane ATPase may lead to a loss of its function and consequently to a decrease in sperm motility. This outcome was also confirmed by Kumosani et al. 2008, who observed that men smokers have a diminution of Ca^{2+}-ATPase and simultaneously a decrease in sperm motility. If Cd affects the Ca^{2+} dynamics in sperm tail, it is possible that it also interfere with the

axonemal movement, as already described in bovine spermatozoa (Kanous et al. 1993). Oxidative stress is known to induce lipid peroxidation of spermatozoa plasma membrane, which consequently leads to decreased sperm motility and tail and midpiece defects (Elshal et al. 2009). Recently, a proteomic study revealed that human spermatozoa have the capacity to produce ATP through membrane fatty acids (Amaral, Castillo, et al. 2013). Since spermatozoa is vulnerable to lipid peroxidation due to the high content in polyunsaturated fatty acids (Guerriero et al. 2014), this pathway to obtain energy for sperm motility would be compromised. Tail defects may also be related to the excessive production of ROS, which leads to the oxidation of outer dense fibers and fibrous sheath, enriched in sulphydryl groups (Yeung et al. 2009). Mitochondrial DNA may also be damaged by RS, limiting ATP production and energy provision for spermatozoa motility (Wright et al. 2014).

Regarding the accessory gland function, it was only observed a diminution in the total concentration of neutral α-glucosidase (NAG) per ejaculate in TP3 in relation to TP2 and TP1. NAG is secreted by the epididymal epithelium, possibly to provide ideal levels of energy for sperm maturation (Elzanaty et al. 2002). A decrease of this biomarker in TP3 may suggest a long-term effect of alcohol and nicotine consumption in epididymal function. Curiously, previous studies demonstrated that men smokers have a marked reduction in α-glucosidase (Mosad et al. 2003), which in turn may be associated with a defective sperm maturation and consequently to a decrease in sperm motility. Furthermore, lower levels of this enzyme was found in men with oligozoospermia and azoospermia of primary testicular origin (Mahmoud et al. 1998).

In this study, the impact of the lifestyle alterations was also evaluated in three different ways, beginning with the assessment of the total antioxidant status (TAS) of spermatozoa and measuring the expression of two important antioxidant enzymes (SOD1 and GPx4), which have distinct roles in spermatozoa defense. Lastly, the presence of specific alterations at the protein level (carbonyl and 3-nitrotyrosine groups) was also determined.

Total antioxidant capacity of seminal plasma have been studied (Agarwal & Saleh 2002; Benedetti et al. 2012; Agarwal, Durairajanayagam, et al. 2014), with little information concerning the impact of OS on total antioxidant capacity of the sperm cells. The concentration of antioxidants in spermatozoa was measured using a commercial test for TAS determination, which is based on the incubation of ABTS with a peroxidase and H_2O_2 (previously described in Methods section). Although this assay is used to determine the total antioxidant status of the

cells, the RS present in the reaction is H_2O_2 and, therefore, this assay only determine the activity of the antioxidants that interacts with this molecule. In sperm cells, the antioxidant enzymes that may be related to the elimination of H_2O_2 are GPx, catalase and peroxiredoxins (O'Flaherty 2014). In the present study, a significant increase in TAS was observed in TP3 compared to TP1. This increase along the 3 TPs may be explained as an adaptation mechanism due to the increase in the oxidative stress in the sperm cells (Prior & Cao 2001).

Immunocytochemical analysis was also performed to determine the localization of the antioxidant enzymes SOD1, GPx1 and GPx4. Only SOD1 immunolocalization was previously detected in human sperm cells, using immunocytochemistry analysis, and it was found in principal piece, the midpiece, and posterior part of the head, excluding the nucleus and the acrosome (Lasso et al. 1994). In this study, SOD1 immunoreactivity was observed in the head, midpiece and the entire length of the tail. In the same way, immunoreactivity for GPx1 and GPx4 was also found in these sperm compartments, predominantly in the midpiece, particularly in the case of GPx4. These results are in agreement with the literature (Chabory et al. 2010; Koppers 2012; O'Flaherty 2014).

SOD is an important enzyme responsible for the conversion of $O_2^{\cdot -}$ into H_2O_2 (Tremellen 2008). Although 3 isoforms of SOD exist, in human spermatozoa, SOD1 plays a major role in the removal of $O_2^{\cdot -}$. However, besides the SOD1, seminal plasma is also well equipped with extracellular SOD (SOD3) (O'Flaherty 2014). The production of H_2O_2 is subsequently removed due to the action of GPxs to H_2O and O_2 (Aitken & Roman 2008). The role of GPxs to the protection against ROS is limited, since human spermatozoa, testis and seminal plasma do not have GPx2, GPx3 and GPx5 (O'Flaherty 2014). It was previously shown that GPx4 is found as a soluble and enzymatically active peroxidase in spermatids, but in mature spermatozoa the mitochondrial isoform is enzymatically inactive and only plays a structural role (Ursini et al. 1999). Taking account the immunolocalization, above described, and their biological role, it was analyzed the expression of SOD1 and GPx4, using β-tubulin as a loading control. In this study, we observed a decrease in the expression of these antioxidant enzymes along the TPs studied. However, these alterations were not statistically significant.

The generation of ROS, such as $O_2^{\cdot -}$ and H_2O_2, may also lead to the damage of proteins (Dalle-Donne et al. 2003). One of the protein modifications that occur in the presence of ROS is protein carbonylation (Agarwal, Durairajanayagam, et al. 2014). The formation of carbonyl groups occurs in the protein side chains, mainly in Pro, Arg, Lys and Thr residues, when they

are oxidized (Stocker et al. 2015). These groups may also be produced by the reaction between the side chains of proteins and aldehydes produced during lipid peroxidation, such as 4-HNE and MDA. Since carbonyl groups are very stable (Dalle-Donne et al. 2003), they are frequently used as OS biomarkers. We did not find statistically significant differences between the TPs studied, although a slight increase in TP2 has been observed. However, in other study, an increase in spermatozoa and seminal plasma protein carbonyls in men smokers was observed and it was positively correlated with seminal plasma Cd levels (Kiziler et al. 2007). Additionally, a negative correlation between the percentage of normal morphological forms and carbonyl groups was also demonstrated (Shiva et al. 2011).

Other protein modification studied in this work was 3-NT formation. 3-NT is produced by the nitration (addition of a NO_2 group) of protein tyrosine residues (**Figure 24**) (Radi 2004). Initially, nitric oxide (NO) is produced from L-arginine through nitric oxide synthase (NOS). In testis and spermatozoa, 3 isoforms of NOS are found: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) (Doshi et al. 2012). Subsequently, the NO produced in the sperm cells may react with $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$), which attacks protein tyrosine residues to produce 3-NT (Morielli & O'Flaherty 2014). Generally, $ONOO^-$ are produced only when NOS has reached toxic levels and SOD cannot compete for the scavenging of $O_2^{\cdot -}$ (Doshi et al. 2012). As $ONOO^-$ is short lived and difficult to quantify directly, the detection of 3-NT is used as biomarker for the formation of this molecule *in vivo* (Halliwell & Poulsen 2006).

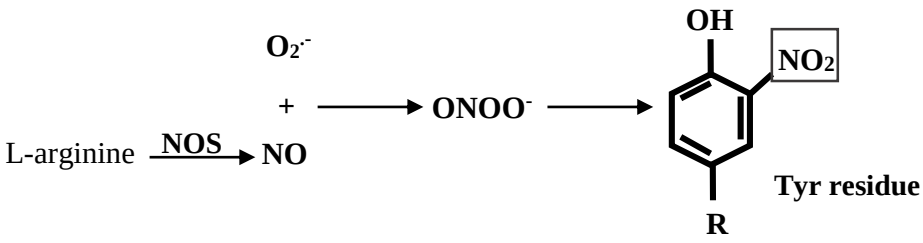

Figure 24 – Formation of 3-nitrotyrosine. NO is produced from L-arginine through NOS. Subsequently, the NO produced in the sperm cells may react with $O_2^{\cdot -}$ to form $ONOO^-$, which attacks protein tyrosine residues to produce 3-NT. **NOS**: nitric oxide synthase; **NO**: nitric oxide; **$O_2^{\cdot -}$**: superoxide anion; **$ONOO^-$**: peroxynitrite; 3-NT: 3-nitrotyrosine

An increase of 3-NT occurred in TP2. However, a marked decrease was observed in TP3. NO and $ONOO^-$ are the major reactive nitrogen species present in tobacco smoking (Halliwell & Poulsen 2006). In the same way, it is known that alcohol consumption accelerates NO

production by NOS (Emanuele & Emanuele 2001). Therefore, an abusive consumption of these substances during academic festivities week may lead to an overproduction of $ONOO^-$ and, consequently, to an increase in protein nitration. A recent study incubated human spermatozoa with a molecule that produces $ONOO^-$ and observed a decrease in sperm progressive motility and mitochondrial membrane potential, being responsible for the impairment of sperm function (Uribe et al. 2015). Furthermore, it was demonstrated that normospermic men have lower levels of $ONOO^-$ than asthenozoospermic infertile men and a negative correlation between $ONOO^-$ concentration and the percentage of motile spermatozoa was observed (Vignini et al. 2006). Post-translational modifications of proteins, such as protein tyrosine nitration, may have negative effects on a protein's structure, intracellular compartmentalization and catalytic activity. ONOO- also attacks protein sulphydryl groups, affecting its function (Herrero et al. 2001). Since outer dense fibers and fibrous sheath are enriched in sulphydryl groups (Yeung et al. 2009), $ONOO^-$ may also contribute to the impairment of sperm motility, which was already observed *in vitro* (Öztezcan et al. 1999).

After the individual analysis of all assays, we evaluated the possible relations between the consumption of alcohol and nicotine, basic semen parameters, accessory glands function and the studied proteins in a total of 45 samples. In **Table 19**, all the correlations verified with the Pearson and Spearman test are represented.

Relatively to alcohol and nicotine consumption, only alcohol revealed a positive correlation with 3-NT content and a negative correlation with fructose concentration and total concentration of fructose per ejaculate. NOS are found in seminal vesicles, where fructose is produced (Gonzales 2001). As previously described, alcohol accelerates nitric oxide production by NOS (Emanuele & Emanuele 2001) and, consequently, may lead to an excessive production of $ONOO^-$ and an increase in 3-NT formation. Since alcohol consumption seems to affect accessory glands function, particularly seminal vesicles, a reduction in the percentage of motile spermatozoa may occur, since fructose is a source of energy for sperm metabolism (Andrade-Rocha 2003).

Subsequently, it was evaluated the relationship between OS parameters and the basic semen parameters. A positive correlation between 3-NT and midpiece defects and age was found. 3-NT is a biomarker of the *in vivo* production of $ONOO^-$, which can react and inactivate many proteins, including GPxs (Halliwell & Poulsen 2006). Since GPx4 plays a major role in normal formation of the mitochondrial sheath in midpiece region (Chabory et al. 2010; O'Flaherty 2014), alterations and/or the inactivation of this protein induced by 3-NT may have

negative consequences for mitochondria helix structure and, consequently, leads to structural defects in this sperm compartment. Additionally, increased concentrations of 3-NT have been documented in both normal aging as well as in some pathologies, such as Alzheimer and Parkinson's disease (Beal 2002), which could explain the positive correlation obtained.

A positive and a negative correlation between carbonyl groups and immotility and progressive motility was respectively found. The same relationship was also verified in other studies (Saraniya et al. 2008; El-Taieb et al. 2009). El-Taieb and colleagues also found a positive correlation between carbonyl groups and axonemal anomalies, which explain the diminution or loss of the sperm motility.

Semen volume positively correlates with carbonyl groups and GPx4. The increase in sexual abstinence is one of the causes of the increase in semen volume, increasing 0,4mL each day (Potts 2012). Furthermore, prolonged abstinence time may induce leukocytospermia (Patton & Battaglia 2005; Blackwell & Zaneveld 1992). Leukocytes are one of the major sources of RS in seminal plasma (Agarwal, Virk, et al. 2014), which can cause an increase in protein modifications, such as the addition of carbonyl groups in proteins. In an attempt to eliminate RS produced by leukocytes, an increase in GPxs may occur, which may explain the positive correlation between semen volume and GPx4 and carbonyl groups.

In the other hand, a negative correlation between sperm concentration and TAS and SOD1 expression was observed. This might be explained by an increase in OS during spermatogenesis, which leads to an increase in total antioxidant capacity of the cells, including an increase in SOD1 expression, to eliminate the RS generated. Consequently, an increase in OS may lead to the apoptosis of sperm cells, which may justify the decrease of sperm concentration. However, these results are contradictory with the studies developed by Shiva and colleagues that found a positive correlation between SOD and sperm count (Shiva et al. 2011).

Table 19 - Overview of the correlated parameters evaluated in the "*Para o Frasco*" study.

	Citric acid [] (mg/mL)	**Total citric acid (mg)**	**Fructose [] (mg/mL)**	**Total fructose (mg)**	**NAG [] (mg/mL)**	**Total NAG (mg)**	**TAS (mmol/L)**	**SOD1**	**GPx4**	**CG**	**3-NT**
Age (years)					r_s=-0,31* N=43						r_s=0,326* N=45
Sexual abstinence (days)	r_s=0,329* N=44										
Volume (mL)		r_s=0,515** N=45		r_s=0,493** N=43	r_s=-0,366* N=43	r_s=0,371* N=45			r_s=0,352* N=42	r_s=0,349* N=42	
Sperm [ ] (x10^6 spz/mL)	r_s=0,45* N=44				r_s=0,333* N=43	r_s=0,328* N=45	r_s=-0,335* N=42	r_s=-0,36* N=42			
Total sperm [ ] (x10^6 spz)		r_s=0,481** N=45		r_s=0,461** N=43		r_s=0,488** N=45					
Progressive motility (%)										r_s=-0,312* N=41	
Immotility (%)										r_s=0,334* N=41	
Normal sperm (%)						r_s=0,322* N=40					
Head defects (%)	r=-0,399** N=41										
Midpiece defects (%)											r=0,309 N=41
TAS (mmol/L)			r_s=-0,387* N=40		r_s=-0,377* N=40			r_s=0,339* N=42	r_s=0,34* N=42		
SOD1 expression							r_s=0,339* N=42		r_s=0,677** N=42		
GPx4 expression	r_s=-0,36* N=41			r_s=0,343* N=40			r_s=0,34* N=42	r_s=0,677** N=42			
3-NT					r_s=-0,347* N=43						
Alcohol consumption (g)			r_s=-0,392** N=43	r_s=-0,339* N=43							r_s=0,365* N=45

NAG: neutral α-glucosidase; **TAS**: total antioxidant status; **SOD1**: superoxide dismutase 1; **GPx4**: glutathione peroxidase 4; **CG**: carbonyl groups; **3-NT**: 3-nitrotyrosine; **spz**: spermatozoa

The relationship between basic semen parameters and accessory gland function was also assessed. Semen volume is composed by seminal vesicles and prostate gland secretions, with a small amount from the bulbourethral glands and epididymis (WHO 2010). A positive correlation between semen volume and total concentration of citric acid, fructose and NAG was found. Since semen volume is the result of the production of these substances by prostate, seminal vesicles and epididymis, respectively, an increase in their production results in an increase in semen volume. However, a negative correlation was found for NAG concentration. It was also obtained a positive correlation between sexual abstinence time and citric acid concentration. As long periods of sexual abstinence leads to an increase in semen volume and the latter is mainly contributed by seminal vesicles and prostate gland, it is expected to occur an increase in citric acid production with longer sexual abstinence times.

Moreover, a positive correlation was observed between total sperm concentration in ejaculate and the total concentration of citric acid, fructose and NAG. In the same way, a positive correlation was found between sperm concentration and citric acid and NAG concentration and total concentration of NAG per ejaculate. In normal ejaculates, without duct obstruction and when the sexual abstinence time is short, the total number of spermatozoa in the ejaculate is correlated with volume (WHO 2010), which is in agreement with obtained results.

A negative correlation was observed between the age of volunteers and the concentration of NAG. Curiously, the same result was previously described, where it was observed a decrease of NAG concentration with age. This might be explained by the morphological and physiological changes that may occur with aging, which may lead to the decrease in epididymis function (Molina et al. 2010)

Lastly, a negative correlation between acid citric concentration and sperm head defects was found, as well as a positive correlation between NAG concentration and the percentage of spermatozoa with normal morphology. These results may suggest a role of citric acid and neutral α–glucosidase in sperm morphology, more exactly in sperm head defects and normal morphology. A positive correlation between NAG and normal sperm morphology was also observed in a previous study (Mahmoud et al. 1998).

Subsequently, we also assessed the relationship between the OS parameters studied. A strong positive correlation was obtained between SOD1 and GPx4. SOD is responsible by the conversion of $O_2^{\cdot-}$ to H_2O_2, which is subsequently converted to H_2O and O_2 by GPx4. Levels of SOD increased leads to an increase in H_2O_2, which may be eliminated by GPx4, leading

consequently to an overexpression of this enzyme. Furthermore, a positive correlation between TAS and SOD1 and GPx4 was also found. In the same way, since TAS corresponds to the concentration of antioxidant enzymes in sperm cells, an increase in SOD1 and GPx4 concentration consequently leads to the increase in the total antioxidant status of the cells.

Finally, the relationship between OS parameters and accessory gland function was also evaluated. Regarding GPx4, a negative and a positive correlations were respectively observed in citric acid concentration and total concentration of fructose. A negative correlation between TAS and fructose and NAG concentration was also found. These results might suggest an influence of fructose, citric acid and NAG concentration in antioxidant defense system. Furthermore, 3-NT negatively correlates with NAG concentration. This might be explained by the modification in protein's structure, intracellular compartmentalization and catalytic activity of NAG that may be induced by 3-NT (Vignini et al. 2006), which in turn may lead to a decrease in the concentration of this protein.

6. Conclusion

OS has been associated with many toxic effects on the male reproductive system as well as in numerous pathologies, such as cancer, diabetes, varicocele, among others (Doshi et al. 2012). However, RS are known to be important for cell signaling events in sperm physiology, which are critical for spermatozoa to acquire fertilizing capacity (Chen et al. 2013). Many lifestyle alterations, such as the excessive consumption of alcohol and tobacco, are known to induce OS, leading to the impairment of cells physiology (Taha et al. 2012). Therefore, the impact of alcohol and nicotine consumption on basic semen parameters, accessory glands function, oxidative balance of cells and in protein damage was studied.

The results showed that the consumption of alcohol and tobacco smoking induced alterations in the oxidative balance and in the physiology of sperm cells. During TP2, a decrease in sperm progressive motility as well as an increase in sperm tail defects and in protein nitration was observed. About 3 months after academic festivities, altered sperm motility and an increase in tail defects remained to be observed, together with a decrease in semen volume. However, the TAS of the sperm cells increased, which led to a decrease in protein oxidation, namely protein nitration.

After evaluating the influence of alcohol and nicotine consumption on sperm cells, the relationship between the seminal quality, accessory gland function and oxidative balance was evaluated. Alcohol consumption led to the increase of 3-NT groups in sperm proteins and to a decrease of fructose concentration, an important source of energy for sperm motility. An increase in OS may lead to the production of 3-NT, which has a positive correlation with midpiece defects. Sperm motility seems to be also negatively affected by the presence of carbonyl groups. In the other hand, citric acid, produced by prostatic gland, seems to positively affect sperm concentration and negatively correlates with head defects, which also suggests that may have a positive role in sperm morphology.

In conclusion, the lifestyle alterations that occurred during the academic week festivities are responsible for a decrease in semen quality, namely in sperm motility and morphology, and by an increase in protein modifications, such as protein nitration. These alterations may consequently lead to a decrease in fertilizing potential.

7. References

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8. Appendix

8.1. Questionnaires

8.1.1. First questionnaire

Estudo sobre a influência dos festejos académicos na qualidade do esperma

Questionário Nº1

Nota: Este inquérito é confidencial e destina-se exclusivamente a ser utilizado neste projeto de investigação científica e estudos dele decorrentes. Os dados relativos ao e-mail e contacto telefónico dos participantes serão apenas utilizados para envio dos resultados do espermograma e para prestar informações relacionadas com as fases seguintes do estudo.

Voluntário Nº

E-mail	
Telemóvel (facultativo)	
Data da recolha	
Hora da recolha	
Hora de receção da amostra	
Hora de realização do espermograma	
Pretende receber o resultado do espermograma por e-mail? (Sim/Não)	

Dados gerais

Idade	
Nº de dias de abstinência sexual	
Doenças relevantes atuais ou passadas (incluir história de tratamentos oncológicos, parotidite com orquite e consumos atuais de medicamentos ou suplementos alimentares)	
Nº de filhos	

Consumos habituais

Tabaco (nº médio cigarros/dia no último mês)	

Álcool (média diária do último mês) – descrever o tipo de bebidas e quantidades respetivas	
Drogas (média diária do último mês) – descrever o tipo de drogas e quantidades respetivas	
Drogas (história de consumo no passado)	

8.1.2. Second Questionnaire

Estudo sobre a influência dos festejos académicos na qualidade do esperma

Questionário Nº 2

Nota: Este inquérito é confidencial e destina-se exclusivamente a ser utilizado neste projeto de investigação científica e estudos dele decorrentes. Os dados relativos ao e-mail e contacto telefónico dos participantes serão apenas utilizados para envio dos resultados do espermograma e para prestar informações relacionadas com as fases seguintes do estudo.

Voluntário Nº

E-mail	
Telemóvel (facultativo)	
Data da recolha	
Hora da recolha	
Hora de receção da amostra	
Hora de realização do espermograma	
Pretende receber o resultado do espermograma por e-mail? (Sim/Não)	

Dados gerais

Idade	
Nº de dias de abstinência sexual	
Doenças relevantes atuais ou passadas (incluir história de tratamentos oncológicos, parotidite com orquite e consumos atuais de medicamentos ou suplementos alimentares)	

Nº de filhos	
Consumos habituais	
Tabaco (nº médio cigarros/dia durante a semana académica)	
Álcool (média diária durante a semana académica) – descrever o tipo de bebidas e quantidades respetivas	
Drogas (média diária durante a semana académica) – descrever o tipo de drogas e quantidades respetivas	
Drogas (média diária durante a semana académica) – descrever o tipo de drogas e quantidades respetivas	

8.1.3. Third questionnaire

Estudo sobre a influência dos festejos académicos na qualidade do esperma

Questionário Nº3

Nota: Este inquérito é confidencial e destina-se exclusivamente a ser utilizado neste projeto de investigação científica e estudos dele decorrentes. Os dados relativos ao e-mail e contacto telefónico dos participantes serão apenas utilizados para envio dos resultados do espermograma e para prestar informações relacionadas com as fases seguintes do estudo.

Voluntário Nº	
E-mail	
Telemóvel (facultativo)	
Data da recolha	
Hora da recolha	
Hora de receção da amostra	
Hora de realização do espermograma	
Pretende receber o resultado do espermograma por e-mail? (Sim/Não)	

Dados gerais	
Idade	
Nº de dias de abstinência sexual	
Doenças relevantes atuais ou passadas (incluir história de tratamentos oncológicos, parotidite com orquite e consumos atuais de medicamentos ou suplementos alimentares)	
Nº de filhos	
Consumos habituais	
Tabaco (nº médio cigarros/no último mês)	
Álcool (média diária no último mês) – descrever o tipo de bebidas e quantidades respetivas	
Drogas (média diária no último mês) – descrever o tipo de drogas e quantidades respetivas	
Drogas (história de consumo no passado)	

8.2. Solutions

8.2.1. Western Blot

10% Sodium Dodecyl Sulfate (SDS):

10% SDS	5g	**Store at RT.**
ddH$_2$O	50mL	

Stock Solution 30% Acrylamide/0.8% Bisacrylamide:

For 1000mL:

Acrylamide	300g	**Store at 4°C protected from light in aluminium foil.**
Bisacrylamide	8g	

Stock Solution 5xUGB:

For 1000mL:

0,6M Tris-HCl	72,68g	**Adjust the pH to 6.8. Bring to the total volume with ddH$_2$O. Store at 4°C.**
ddH$_2$O	700mL	

Stock Solution 4xLGB:

For 1000mL:

1.5M Tris-HCl	181,71g	**Adjust the pH to 8.8. Bring to the total volume with ddH$_2$O. Store at 4ºC.**
0.4% SDS	4g	
ddH$_2$O	750mL	

10% Ammonium Persulfate (APS):

10% APS	0,1g	**Store at 4°C, best if used within one week.**
ddH$_2$O	1mL	

Stock Solution 10x Running Buffer:

For 1000mL:

0,25M Tris-HCl	30,29g	**You can heat to help dissolving the SDS.** **Adjust the pH to 8.3 and store at RT.**
1,92M Glycine	144,13g	
1% SDS	10g	

4x Loading Buffer (LB):

For 5mL:

40% Glycerol	2mL	**Store at RT for short period or 4ºC for longer periods protected from light in aluminium foil.**
250 mM Tris-HCl pH 6.8	1,3mL of 1M Tris-HCl pH 6.8	
8% SDS	0,4g	
2% β-mercaptoethanol	0,1mL	
ddH_2O	1,65mL	
Bromophenol blue	Add according to the color desired	

Stock Solution 10x Transfer Buffer:

For 1000mL:

0,25M Tris-HCl	30,29g	**The pH should be between 8.2-8.4. Store at RT.**
1,92M Glycine	144,13g	

Dilute to 1x Transfer Buffer in a volume correspondent to the total volume minus the volume of methanol needed. Add 20% Methanol before the transfer. Store at 4°C if transfering small gels.

Stock Solution 10xTBS-T:

For 1000mL:

0.2M Tris-HCl	24,23g	**Adjust the pH to 8.9 and store at 4°C**
1.5M NaCl	87,66g	
Bring to the total volume with ddH_2O		
Adjust the pH to 7.5		
0.5% Tween-20	5mL	

5% Blocking Solution:

5% Low Fat Milk or BSA	5g	**Can be stored for a couple of days at 4ºC, best if used fresh.**
1xTBS-T	50mL	

8.2.2. Slot Blot

Tris (2M) with β-mercaptoethanol (18%):

Tris	**3,63g**	**Store at RT.**
β-mercaptoethanol	3,6mL	
ddH_2O	16,4mL	

2,4-Dinitrophenylhydrazine (DNPH; 20mM) in Trifluoroacetic acid (TFA; 10%):

DNPH	0,198g	**Store at RT protected from light in aluminium foil.**
ddH_2O	1mL	
TFA	5mL	
Add the remaining volume of ddH_2O (44mL);		

8.3. Basic semen analysis data

Table 20 – Basic semen analysis parameters of all the volunteers of "Para o Frasco" study.

		Volunteer Number																								
		1			2			3			4			5			6			7			9			
	Time point	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
Basic semen analysis parameters	Sexual abs (days)	9	5	2	4	3	2	2	1	4	0	0	2	2	2	3	6	13	2	2	1	1	5	1	1	
	Volume (mL)	2,5	2,0	1,7	3,5	2,1	2,8	3,0	2,2	1,8	3,2	5,5	6,0	3,0	2,5	2,7	3,8	7,0	3,1	6,5	6,8	5,6	2,5	3,2	2,5	
	Sperm [] ($x10^6$spz/mL)	29	40	57	120	194	113	35	35	31	20	67	29	0	2	4	95	74	56	39	19	24	27	23	28	
	Total sperm [] ($x10^6$ spz)	72,5	80,0	96,9	420,0	407,4	316,4	105,0	77,0	54,3	64,0	368,5	174,0	0,0	5,0	10,8	361,0	518,0	173,6	253,5	129,2	134,4	67,5	73,6	70,0	
	Progr. Motility (%)	37	21	5	56	46	51	49	28	60	62	59	50		3	3	59	15	41	60	2656	40	20	24		
	Non prog. Motility (%)	5	15	20	13	36	38	14	11	16	8	19	18		12	17	9	25	28	7	19	29	17	9	14	
	Imotility (%)	48	64	60	31	17	11	37	51	24	30	22	32		85	80	32	60	31	33	55	15	43	71	62	
	Normal sperm (%)	16	7	8	19	18	18	17	18	15	19	22	13				8	4	13	3	2	3	12	9	10	
	Head defects (%)	68	78	70	71	60	70	63	71	69	79	60	70				80	90	60	90	91	88	70	81	74	
	Midpiece defects (%)	40	50	40	36	41	47	39	38	30	40	42	51				34	40	44	39	51	30	31	34	36	
	Tail defects (%)	18	40	31	15	30	38	18	28	21	29	20	29				12	33	21	18	20	19	22	29	20	

Table 21 - Basic semen analysis parameters of all the volunteers of "Para o Frasco" study.

		Volunteer Number																				
		11			**15**			**18**			**31**			**32**			**34**			**35**		
	Time point	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Basic semen analysis parameters	Sexual abs.(days)	5	1	1	60	8	60	1	2	3	60	8	60	1	1	1	0	1	0	1	2	3
	Volume (mL)	2,5	3,2	2,5	1,0	0,7	1,0	3,6	5,0	3,1	1,0	0,7	1,0	4,0	1,6	1,0	2,5	2,6	1,5	3,6	5,0	3,1
	Sperm [] ($x10^6$spz/mL)	27	23	28	90	35	45	50	61	31	90	35	45	25	46	26	12	27	32	50	61	31
	Total sperm [] ($x10^6$ spz)	67,5	73,6	70,0	90,0	24,5	45,0	180,0	305,0	96,1	90,0	24,5	45,0	100,0	73,6	26,0	30,0	70,2	48,0	180,0	305,0	96,1
	Prog. Motility (%)	40	20	24	49	37	25	30	18	16	49	37	25	39	33	33	6	26	19	30	18	16
	Non prog. Motilit (%)	17	9	14	12	37	25	6	23	11	12	37	25	21	11	11	12	15	16	6	23	11
	Imotility (%)	43	71	62	39	50	50	63	59	73	39	50	50	40	56	56	83	59	65	63	59	73
	Normal sperm (%)	12	9	10	17	9	9	6	8	4	17	9	9	12	10	11	3	10		6	4	4
	Head defects (%)	70	81	74	50	59	67	65	79	79	50	59	67	51	65	59	75	69		65	83	79
	Midpiece defects (%)	31	34	36	49	49	49	43	50	60	49	49	49	39	39	38	40	50		43	69	60
	Tail defects (%)	22	29	20	37	42	44	43	40	51	37	42	44	30	45	40	31	39		43	60	51

8.4. Accessory glands function data

Table 22 – Accessory glands function parameters of all the volunteers of "Para o Frasco" study.

		Accessory glands function																	
		Citric acid [] (mg/mL)			Total citric acid (mg)			Fructose [] (mg/mL)			Total fructose (mg)			NAG [] (mg/mL)			Total NAG (mg)		
Volunteer Number	**Time points**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1		3,91	4,06	5,08	9,77	8,11	8,64	2,58	2,87	2,93	6,45	5,74	4,99	8,51	13,89	13,32	21,27	27,78	22,65
2		2,83	4,23	1,89	9,89	8,89	5,29	8,29	7,35	6,81	29,01	15,44	19,06	13,08	20,04	13,59	45,77	42,08	38,07
3		2,28	3,22	4,07	6,85	7,09	7,12	3,66	2,66	2,12	10,99	5,86	3,71	14,21	9,13	14,58	42,64	20,08	25,52
4		0,00	6,92	4,77	0,00	38,06	28,62		2,27	2,70		12,47	16,23		11,99	8,88	0,00	65,94	53,27
5		5,25	2,28		15,75	5,70	0,00	2,87	1,85		8,61	4,63		8,76	5,72		26,27	14,30	0,00
6		5,77	5,25	4,40	21,91	36,74	13,65	1,95	2,83	3,73	7,43	19,80	11,55	20,50	16,35	9,78	77,90	114,45	30,31
7		3,07	2,60	2,21	19,96	17,69	12,36	1,66	1,60	1,33	10,81	10,88	7,44	10,81	4,22	3,68	70,30	28,66	20,62
9		2,69	2,53	3,98	8,89	7,58	11,94	5,85	6,12	4,75	19,31	18,36	14,24	57,70	46,15	40,91	190,42	138,46	122,74
11		4,85	5,22	5,21	12,11	16,71	13,03	2,75	3,60	3,35	6,87	11,52	8,38	18,13	13,95	13,88	45,33	44,66	34,69
15		8,52	4,31	4,38	25,55	4,31	4,39	2,89	1,60	2,83	8,68	1,60	2,83	15,82	5,97	6,42	47,45	5,97	6,42
18		5,87	7,46	7,54	5,87	5,22	7,54	2,31	2,10	2,73	2,31	1,47	2,73	27,86	17,38	12,08	27,86	12,16	12,08
31		2,69	3,14	3,86	10,21	10,35	4,63	2,02	2,08	2,02	7,66	6,86	2,42	7,61	13,01	17,54	28,92	42,94	21,04
32		4,24	5,13	4,23	16,98	8,20	4,24	2,23	3,04	3,52	8,90	4,86	3,52	13,31	12,93	11,04	53,22	20,69	11,04
34		2,71	3,45	4,25	6,78	8,96	6,38	5,48	3,64	4,06	13,69	9,47	6,09	2,37	17,40	15,86	5,92	45,25	23,79
35		4,24	2,85	3,54	15,28	14,27	10,97	4,77	5,14	5,58	17,16	25,71	17,30	9,37	13,84	8,28	33,72	69,19	25,66

NAG: neutral α-glucosidase

8.5. Antioxidant defense system data

Table 23 – Antioxidant defense system parameters of all the volunteers of "Para o Frasco" study.

		TAS (mmol/L)			SOD1			GPx4		
	Time Point	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Volunteer Number	1	0,43	1,75	2,31	0,78	0,86	0,57	0,11	0,33	0,11
	2	0,92	0,96	2,26	0,76	0,79	0,72	0,60	0,69	0,59
	3	1,32	1,98	2,12	0,81	0,93	0,81	0,31	0,41	0,48
	4	2,00	2,13	2,13	0,75	1,09	1,02	0,51	0,60	0,54
	5	2,64652	2,62472	3,1392	1,980443	2,900196	5,483897	0,405738	2,899168	2,253187
	6	2,16	1,93	2,04	2,71	1,07	1,21	0,31	0,61	0,55
	7									
	9	0,27	0,56	0,72	1,39	2,03	1,13	0,48	0,77	0,43
	11	0,30	0,37	0,07	0,66	0,72	0,80	0,07	0,09	0,04
	15	2,02	0,73	2,04	0,70	0,51	0,12	0,14	0,04	0,03
	18	0,42	0,72	2,18	7,18	0,99	0,98	0,80	0,12	0,19
	31	2,51	2,58	2,52	15,59	4,85	0,93	5,26	1,55	0,29
	32	2,63	2,54	2,40	1,33	1,06	3,98	0,50	0,28	1,41
	34	1,73	1,85	1,87	0,75	1,14	0,98	0,13	0,26	0,29
	35	0,70	1,95	1,74	1,17	0,97	0,91	0,56	0,46	0,48

TAS: total antioxidante status; **SOD1**: superoxide dismutase 1; **GPx4:** glutathione peroxidase 4

8.6. Protein oxidation data

Table 24 – Protein oxidation parameters of all the volunteers of "Para o Frasco" study.

Volunteer Number	Time points	3-nitrotyrosine			Carbonyl groups		
		1	2	3	1	2	3
	1	377,96	410,20	240,21	56,27229	109,3116	68,00704
	2	222,22	136,62	208,82	101,60	137,77	97,60
	3	200,34	314,97	210,46	206,06	204,73	71,02
	4	143,83	297,61	273,19	125,9209	112,8457	122,1431
	5	82,86	282,63	196,97	160,87	135,80	170,42
	6	69,08	176,09	79,38	68,19	110,06	72,82
	7	134,79	252,77	118,04			
	9	91,83	56,11	162,02	63,35	129,97	172,59
	11	210,88	199,10	179,02	62,80	76,69	104,10
	15	199,48	341,27	103,99	157,73	107,74	126,57
	18	73,30	187,28	226,47	117,36	91,78	96,32
	31	176,64	149,57	66,24	125,20	102,97	163,30
	32	383,3254	369,39	268,7163	113,97	96,29	75,23
	34	287,1679	332,4151	333,0316	98,37	239,35	87,02
	35	364,4079	419,3684	250,7809	362,25	392,99	125,34

8.7. Results without statistical significance

8.7.1. Lifestyle alterations

8.7.1.1. Alcohol consumption

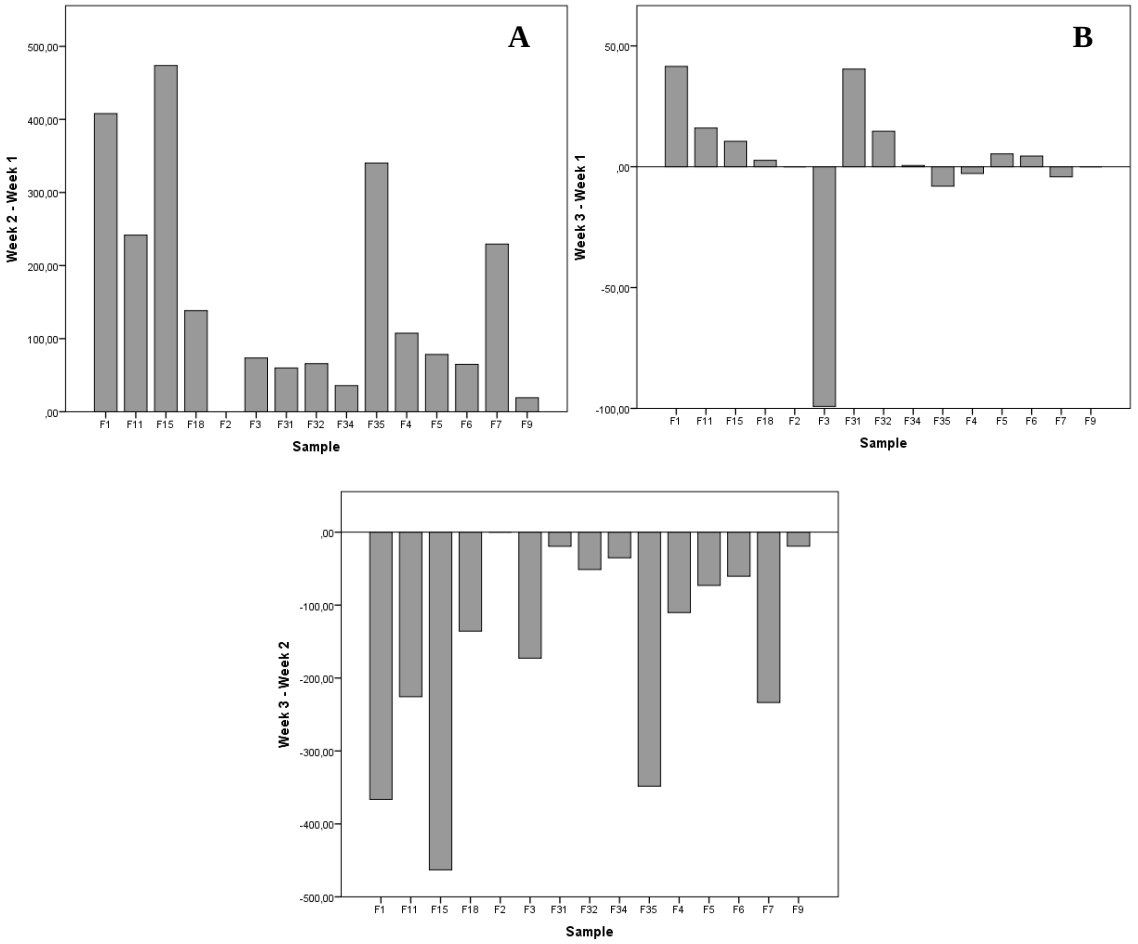

Figure 25 - (A) Bar plot sorted difference between TP2 and TP1 as a function of samples per alcohol consumption; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per alcohol consumption; and **(C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per alcohol consumption.

8.7.1.2. Nicotine consumption

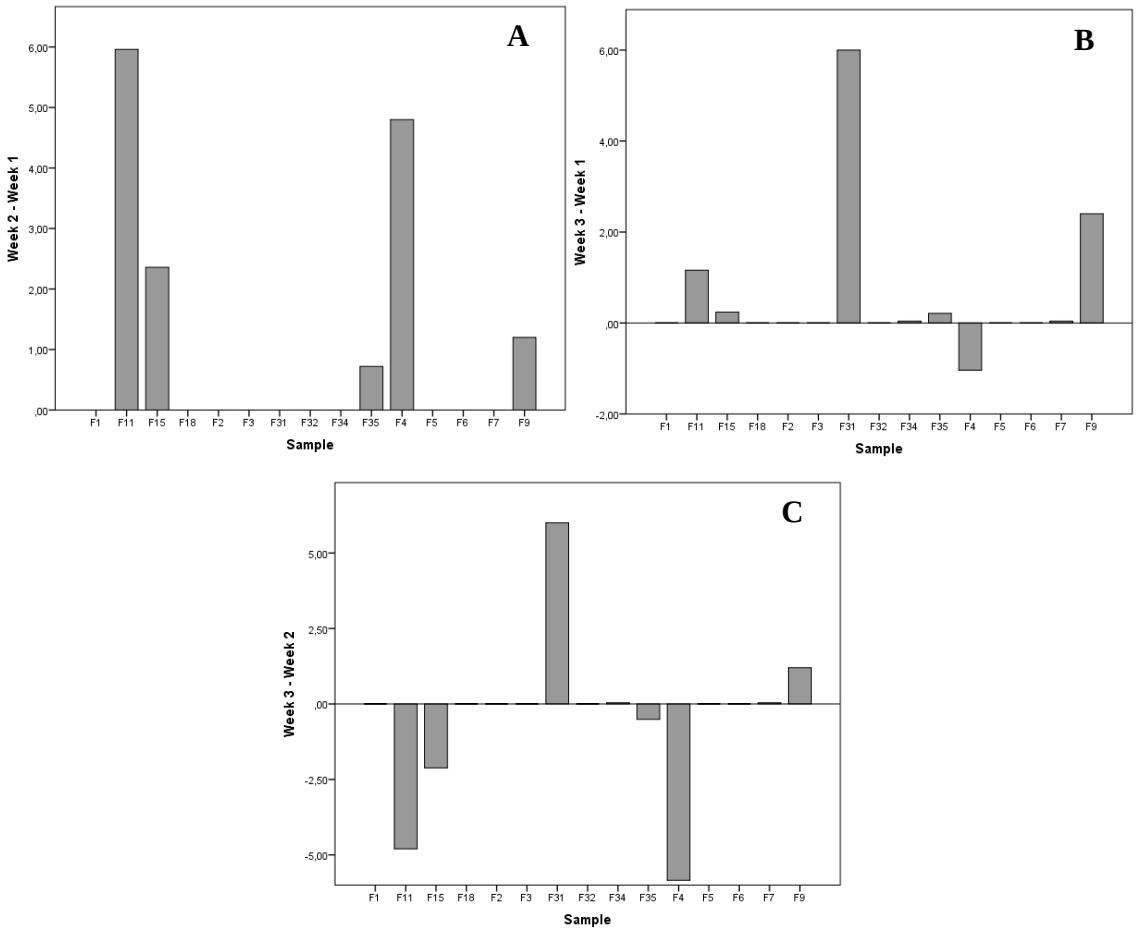

Figure 26 - (A) Bar plot sorted difference between TP2 and TP1 as a function of samples per nicotine consumption; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per nicotine consumption; and **(C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per nicotine consumption.

8.7.2. Basic semen parameters

8.7.2.1. Sexual abstinence

Table 25 – Descriptive statistics of the sexual abstinence. The results are presented in days.

	Sexual abstinence		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	10,6	3,2	12,9
Std. Deviation	20,2	3,7	22,8
Minimum	0	0	0
Maximum	60	13	60

TP: time point

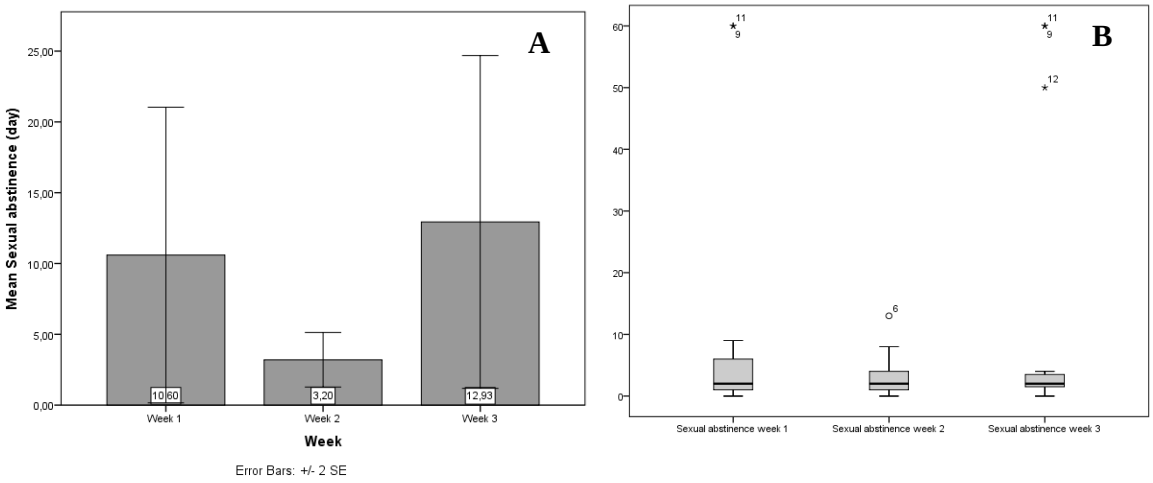

Figure 27 - (A) Bar graph and error bars of sexual abstinence; **(B)** Box plot and outliers of sexual abstinence at the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. For the sexual abstinence, there is no significant difference between the three weeks (p=0,368).

8.7.2.2. Semen volume

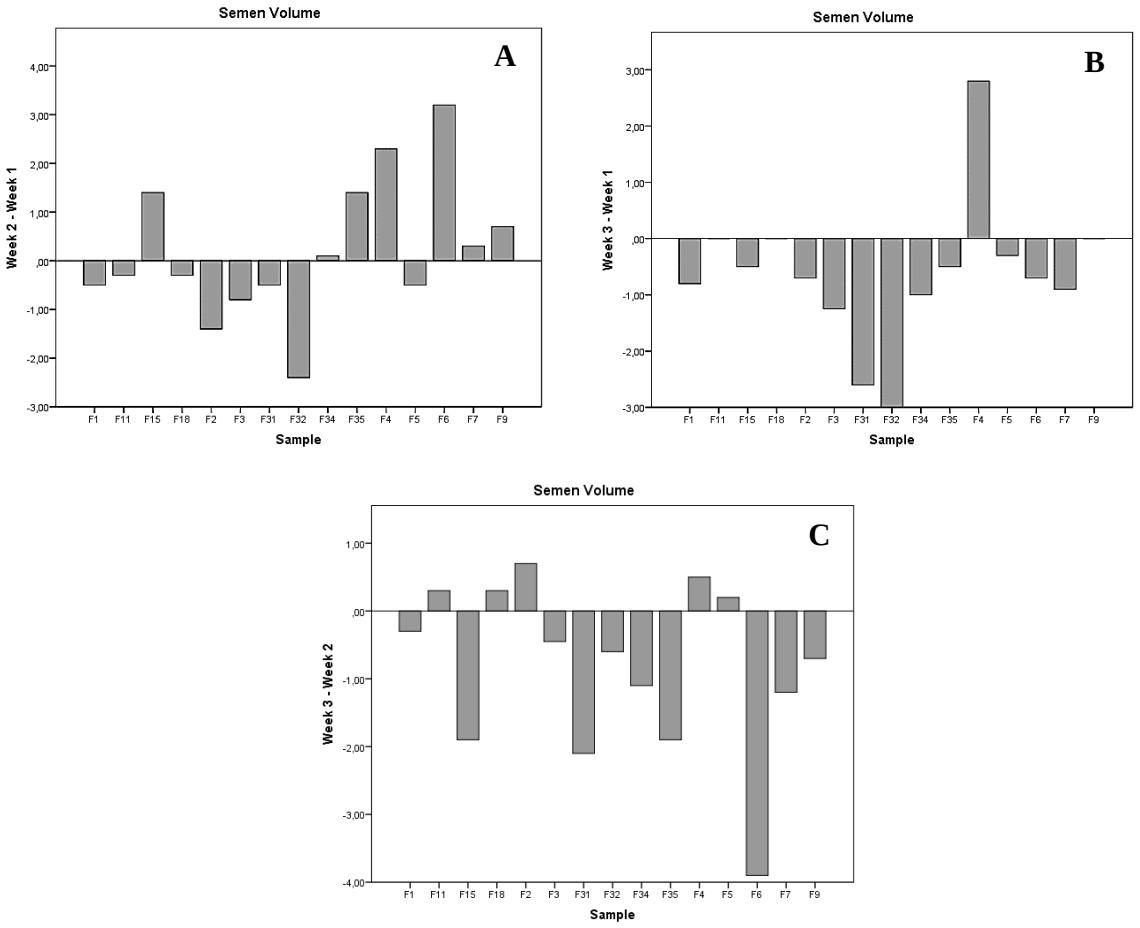

Figure 28 - (A) Bar plot sorted difference between TP2 and TP1 as a function of samples per semen volume; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per semen volume; and **(C)** Bar plot sorted difference between TP3 and TP2 as a function of samples per semen volume.

8.7.2.3. Sperm concentration

Table 26 – Descriptive statistics of the sperm concentration. The results are presented in millions per milliliter.

	Sperm concentration		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	46,80	49,67	38,07
Std. Deviation	35,513	44,506	24,826
Minimum	0	2	4
Maximum	120	194	113

TP: time point

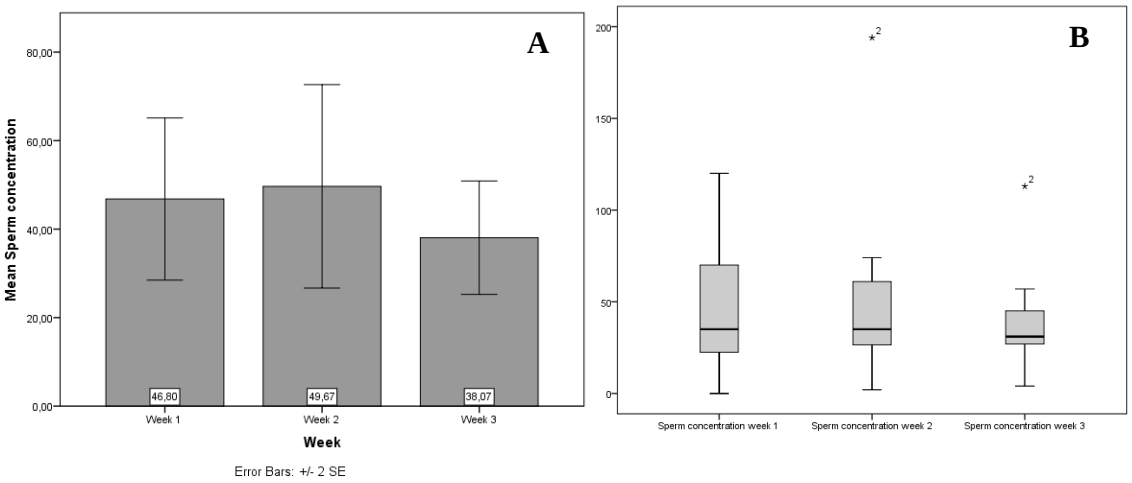

Figure 29 - **(A)** Bar graph and error bars of sperm concentration; **(B)** Box plot and outliers of sperm concentration at the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. For the semen concentration, there is no significant difference between the three weeks (p=0,701).

8.7.2.4. Total number of spermatozoa per ejaculate

Table 27 – Descriptive statistics of the total number of spermatozoa per ejaculate. The results are presented in millions.

	Total number of spermatozoa per ejaculate		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	139,3	169,8	94,0
Std. Deviation	120,3	164,1	80,0
Minimum	,0	5,0	10,8
Maximum	420,0	518,0	316,4

TP: time point

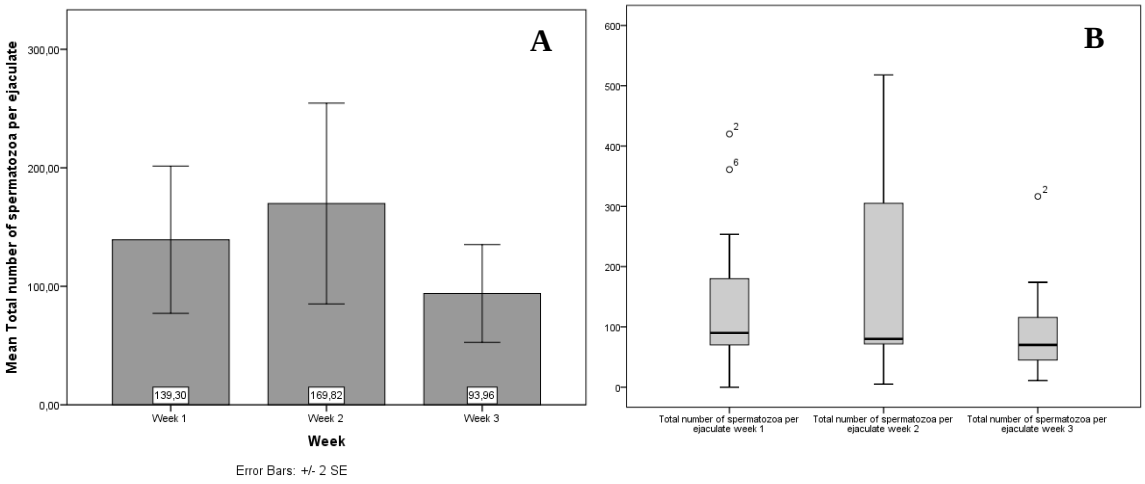

Figure 30 - **(A)** Bar graph and error bars of total number of spermatozoa per ejaculate; **(B)** Box plot and outliers of total number of spermatozoa per ejaculate at the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. For the total number of spermatozoa per ejaculate, there is no significant difference between the three weeks (p=0,247).

8.7.2.5. Progressive motility

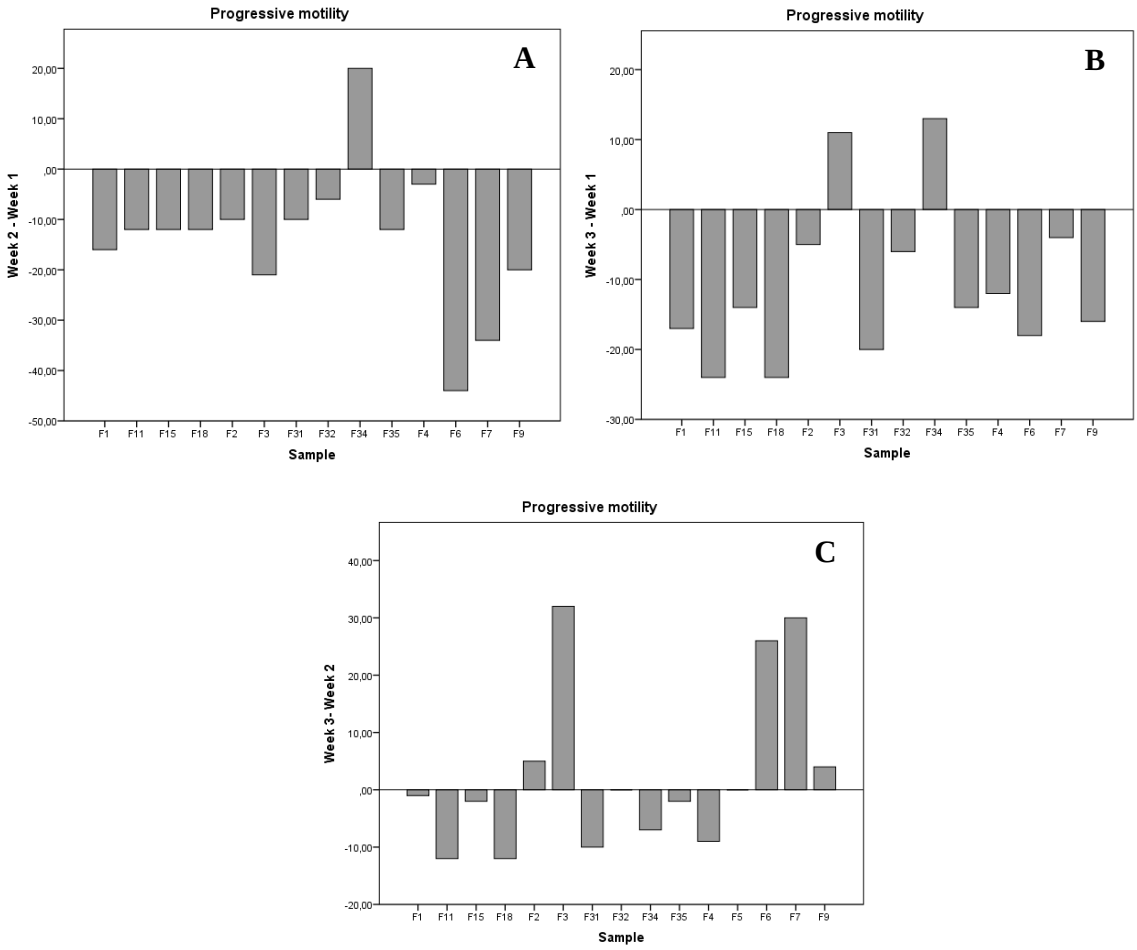

Figure 31 - **(A)** Bar plot sorted difference between TP2 and TP1 as a function of samples per progressive motility; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per progressive motility; and **(C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per progressive motility.

8.7.2.6. Non progressive motility

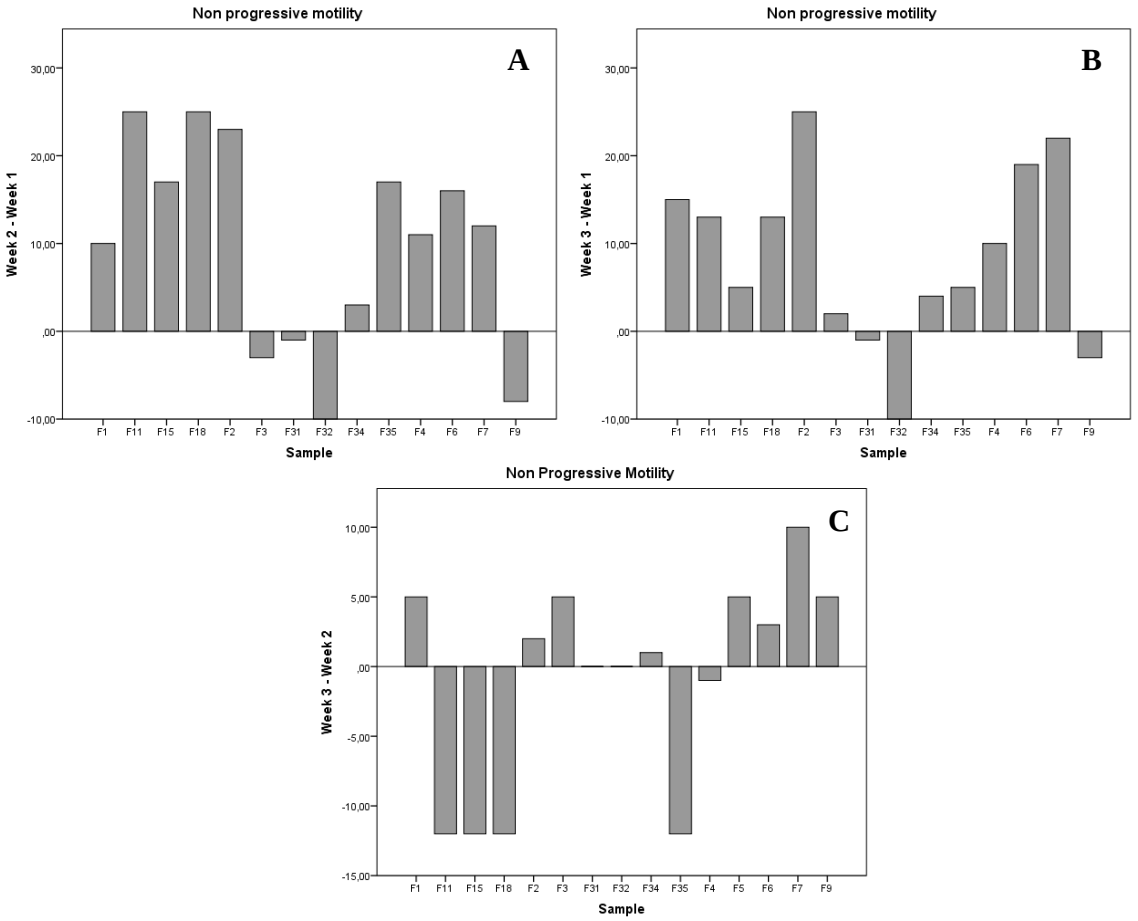

Figure 32 - **(A)** Bar plot sorted difference between TP2 and TP1 as a function of samples per progressive motility; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per progressive motility; and **(C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per progressive motility.

8.7.2.7. Sperm Immotility

Table 28 – Descriptive statistics of the sperm immotility. The results are presented in percentage.

	Immotility		
	TP1	**TP2**	**TP3**
N	14	15	15
Mean	45,79	55,27	50,87
Std. Deviation	15,729	17,248	23,176
Minimum	30	17	11
Maximum	83	85	81

TP: time point

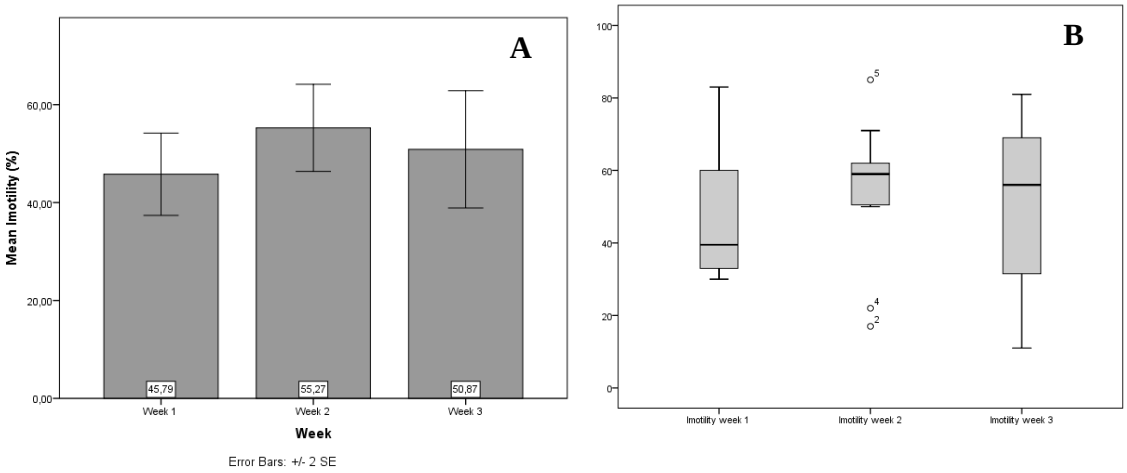

Figure 33 - **(A)** Bar graph and error bars of imotility; **(B)** Box plot and outliers of sperm imotility at the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. For the sperm imotility, there is no significant difference between the three weeks (p=0,397).

8.7.2.8. Normal sperm

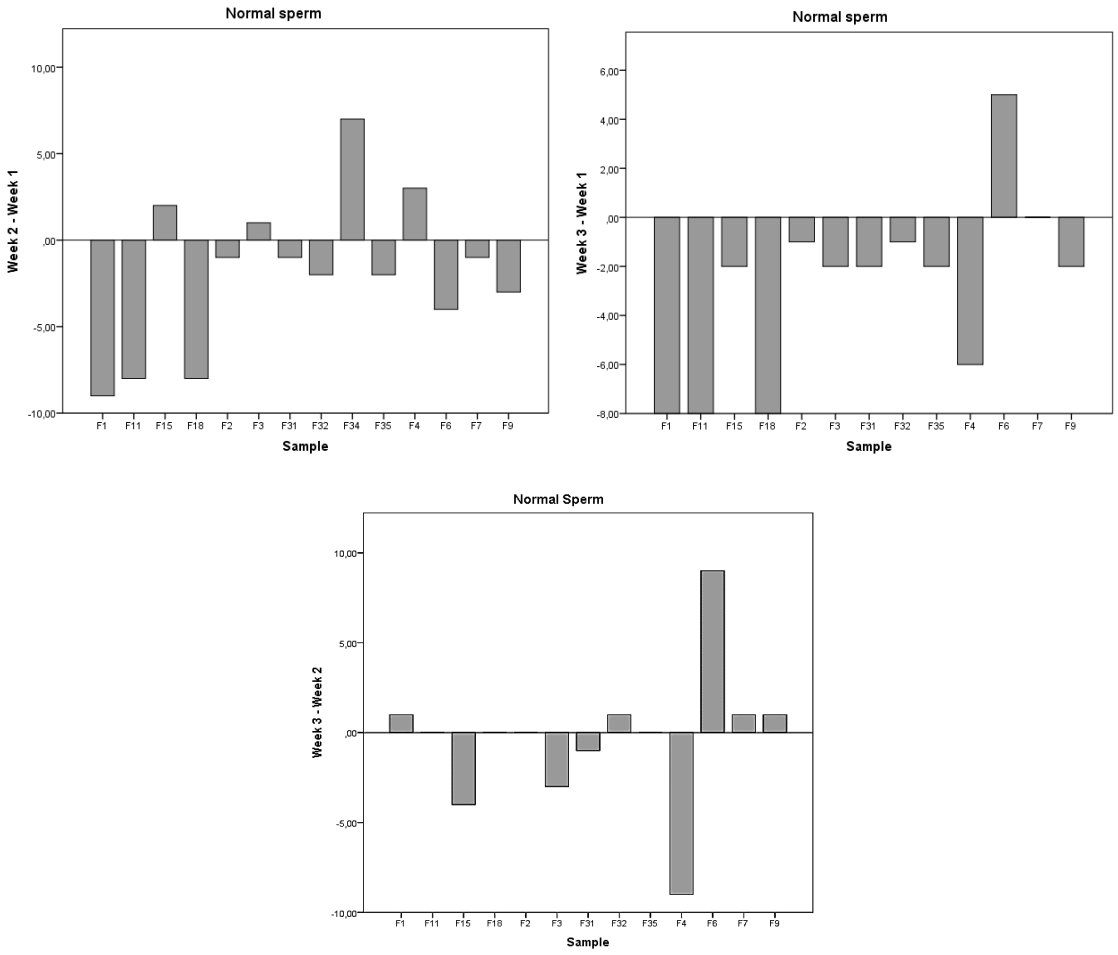

Figure 34 - (A) Bar plot sorted difference between TP2 and TP1 as a function of samples per normal sperm; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per normal sperm; and **(C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per normal sperm.

8.7.2.9. Sperm head defects

Table 29 – Descriptive statistics of the sperm head defects. The results are presented in percentage.

	Head defects		
	TP1	**TP2**	**TP3**
N	14	14	13
Mean	66,9	72,6	71,5
Std. Deviation	11,9	11,3	8,0
Minimum	50	59	59
Maximum	90	91	88

TP: time point

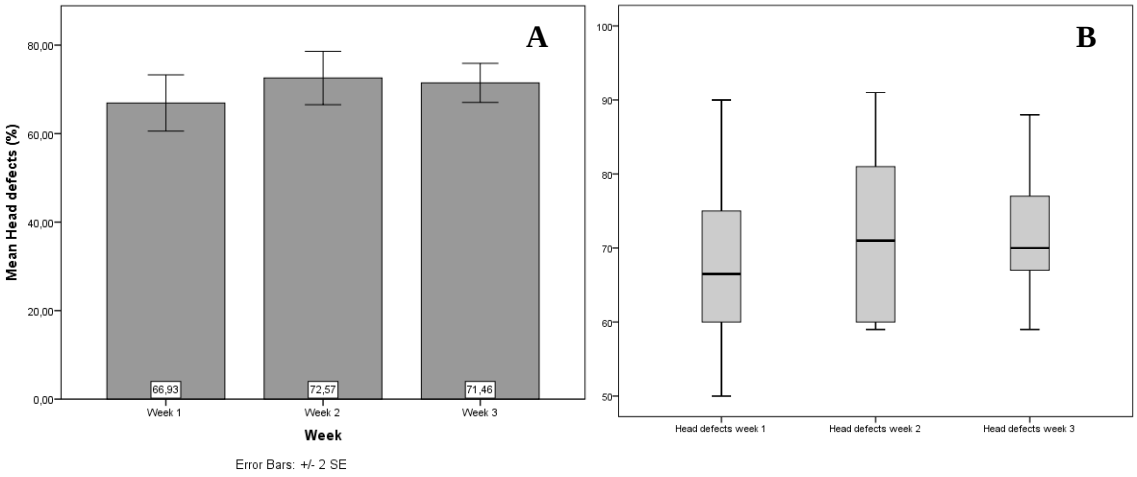

Figure 35 - (A) Bar graph and error bars of head defects; **(B)** Box plot and outliers of sperm head defects at the three weeks.

An ANOVA with repeated measures was performed to determine if the differences between the three weeks were statistically significant. For the head defects, there is no significant difference between the three weeks (p=0,128).

8.7.2.10. Sperm midpiece defects

Table 30 – Descriptive statistics of the sperm midpiece defects. The results are presented in percentage.

	Midpiece defects		
	TP1	**TP2**	**TP3**
N	14	14	13
Mean	40,8	46,7	43,7
Std. Deviation	5,5	8,7	10,1
Minimum	31	34	30
Maximum	49	69	60

TP: time point

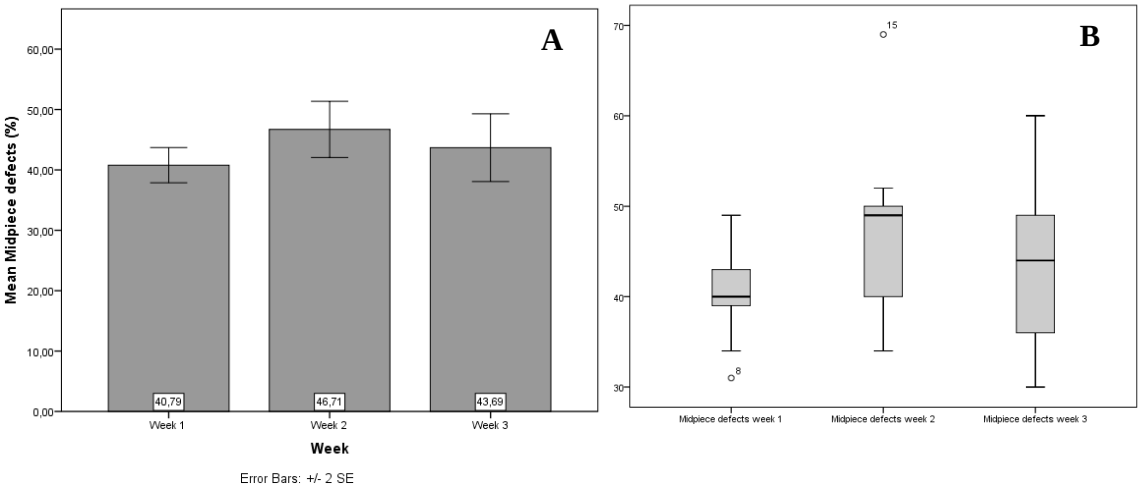

Figure 36 - (A) Bar graph and error bars of midpiece defects; **(B)** Box plot and outliers of sperm midpiece defects at the three weeks.

An ANOVA with repeated measures was performed to determine if the differences between the three weeks were statistically significant. As for the midpiece defects, there is no significant differences between the three weeks (p=0,066).

8.7.2.11. Tail defects

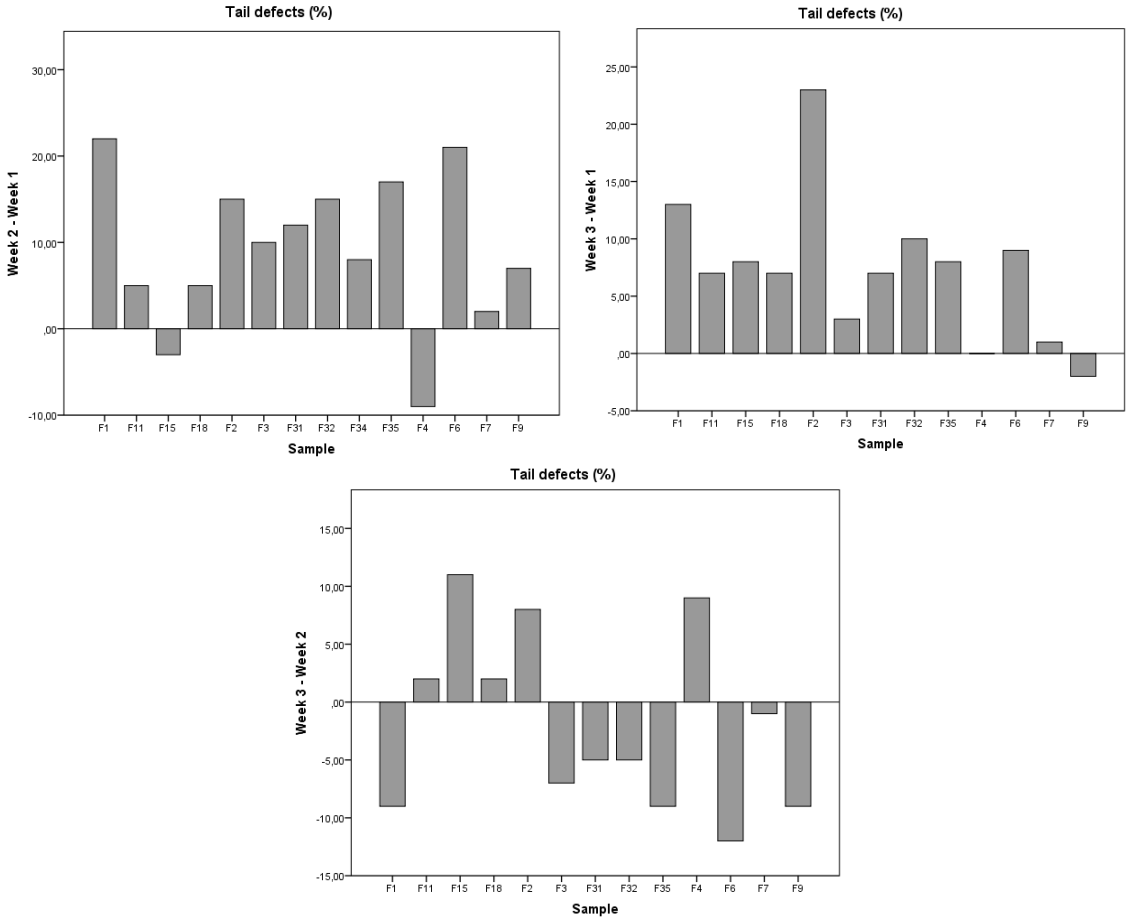

Figure 37 - (A) Bar plot sorted difference between TP2 and TP1 as a function of samples per tail defects; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per tail defects; and (**C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per tail defects.

8.7.3. Accessory gland function

8.7.3.1. Citric acid concentration

Table 31 - Descriptive statistics of the citric acid concentration. The results are presented in milligrams per milliliter.

	Citric acid concentration		
	TP1	**TP2**	**TP3**
N	15	15	14
Mean	3,93	4,18	4,24
Std. Deviation	1,99	1,57	1,34
Minimum	0,00	2,28	1,89
Maximum	8,52	7,46	7,54

TP: time point

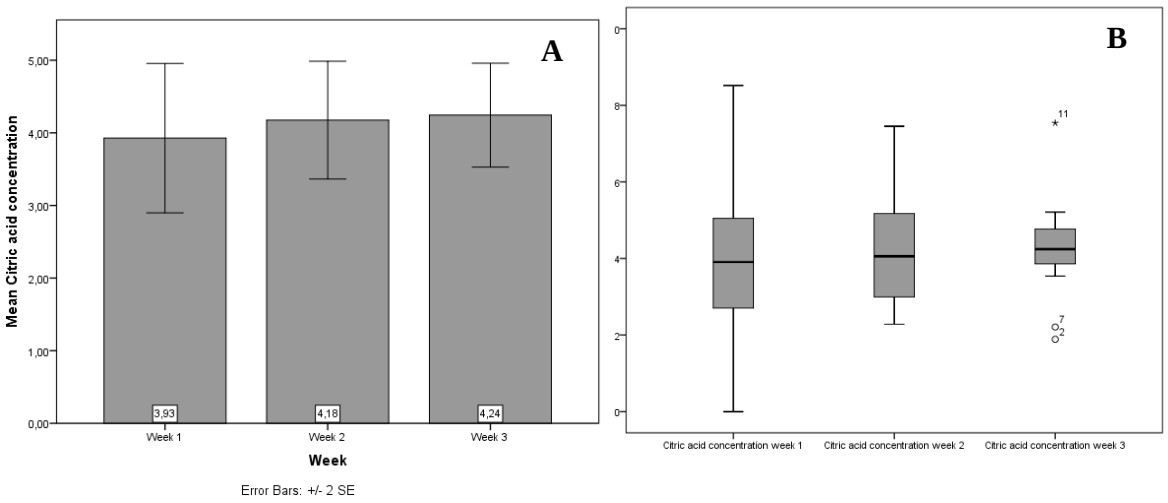

Figure 38 - **(A)** Bar graph and error bars of citric acid concentration; **(B)** Box plot and outliers of citric acid concentration at the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. For the citric acid concentration, there is no significant differences between the three weeks (p=0,607).

8.7.3.2. Total concentration of citric acid per ejaculate

Table 32 – Descriptive statistics for the total concentration of citric acid per ejaculate. The results are presented in milligrams per ejaculate.

	Total citric acid concentration		
	TP1	**TP2**	**TP3**
N	15	15	15
Mean	12,38553	13,19200	9,25213
Std. Deviation	6,814354	10,587368	6,634674
Minimum	,000	4,310	,000
Maximum	25,549	38,061	28,620

TP: time point

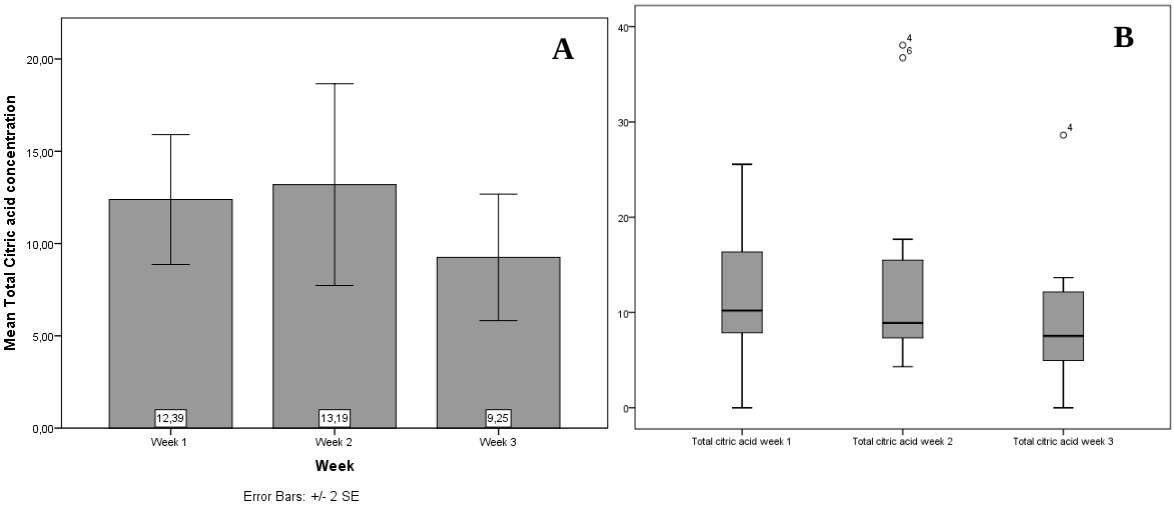

Figure 39 - **(A)** Bar graph and error bars of total concentration of citric acid per ejaculate; **(B)** Box plot and outliers of total concentration of citric acid per ejaculateat the three weeks.

An ANOVA with repeated measures was performed to determine if the differences between the three weeks were statistically significant. There is no significant differences between the three weeks (p=0,072).

8.7.3.3. Fructose concentration

Table 33 – Descriptive statistics of the fructose concentration. The results are presented in milligrams per milliliter.

	Fructose concentration		
	TP1	**TP2**	**TP3**
N	14	15	14
Mean	3,52	3,25	3,46
Std. Deviation	1,90	1,70	1,47
Minimum	1,66	1,60	1,33
Maximum	8,29	7,35	6,81

TP: time point

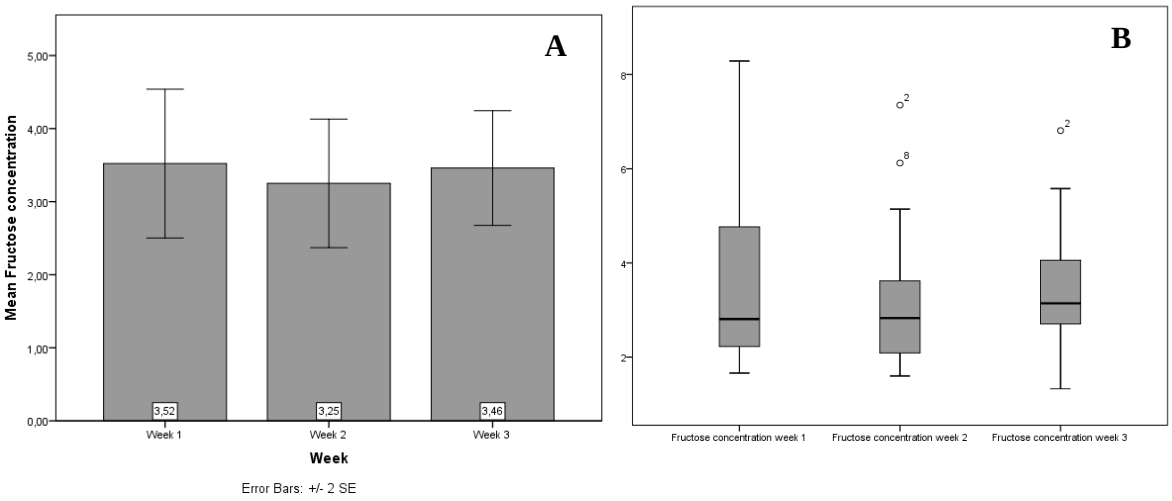

Figure 40 - **(A)** Bar graph and error bars of fructose concentration; **(B)** Box plot and outliers of fructose concentration at the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. For the fructose concentration, there is no significant differences between the three weeks (p=0,981).

8.7.3.4. Total concentration of fructose per ejaculate

Table 34 – Descriptive statistics of total concentration of fructose per ejaculate. The results are presented in milligrams per ejaculate.

	Total fructose concentration		
	TP1	**TP2**	**TP3**
N	14	15	14
Mean	11,28	10,31	8,60
Std. Deviation	6,74	7,04	5,95
Minimum	2,31	1,47	2,42
Maximum	29,01	25,71	19,06

TP: time point

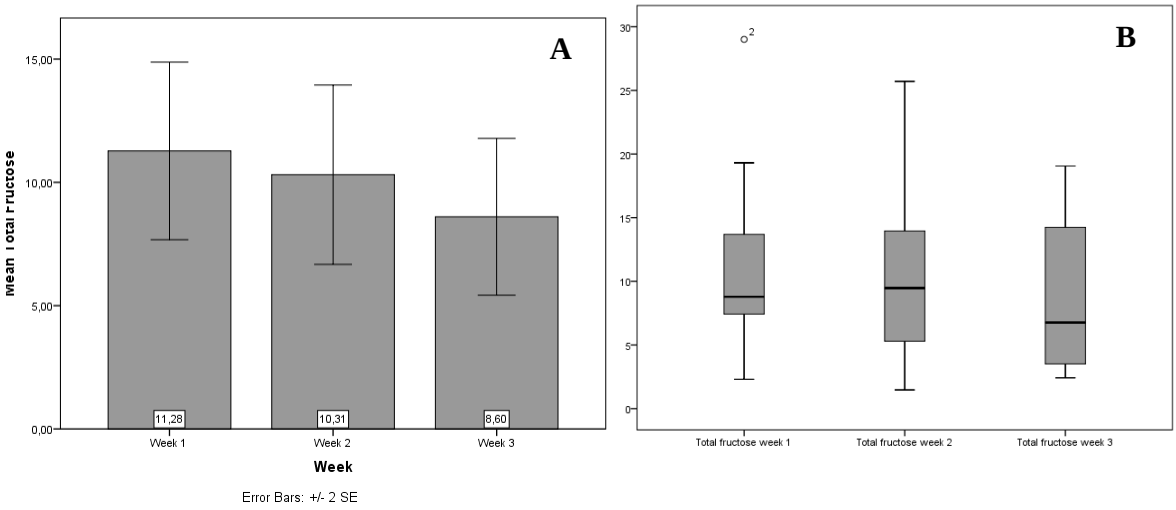

Figure 41 - (A) Bar graph and error bars of total concentration of fructose per ejaculate; **(B)** Box plot and outliers of total concentration of fructose per ejaculate at the three weeks.

A Friedman test to determine if the differences between the three weeks were statistically significant. There is no significant differences between the three weeks (p=0,092).

8.7.3.5. NAG concentration

Table 35 – Descriptive statistics of the NAG concentration. The results are presented in milligrams per milliliter.

	NAG concentration		
	TP1	**TP2**	**TP3**
N	14	15	14
Mean	16,29	14,80	13,56
Std. Deviation	13,47	9,81	8,73
Minimum	2,37	4,22	3,68
Maximum	57,70	46,15	40,91

TP: time point

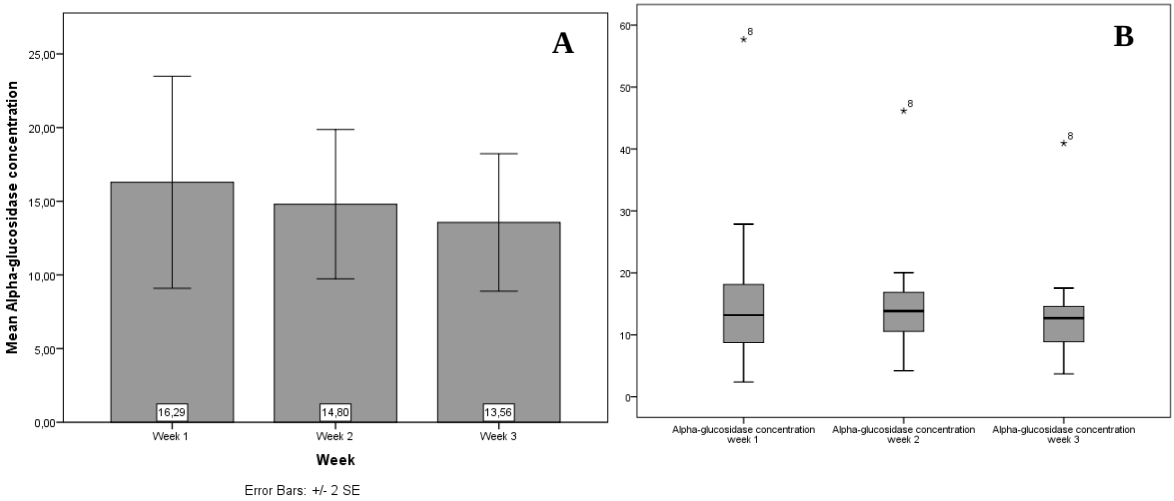

Figure 42 - **(A)** Bar graph and error bars of NAG concentration; **(B)** Box plot and outliers of NAG concentration the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. There is no significant differences between the three weeks (p=0,232).

8.7.3.6. Total concentration of NAG per ejaculate

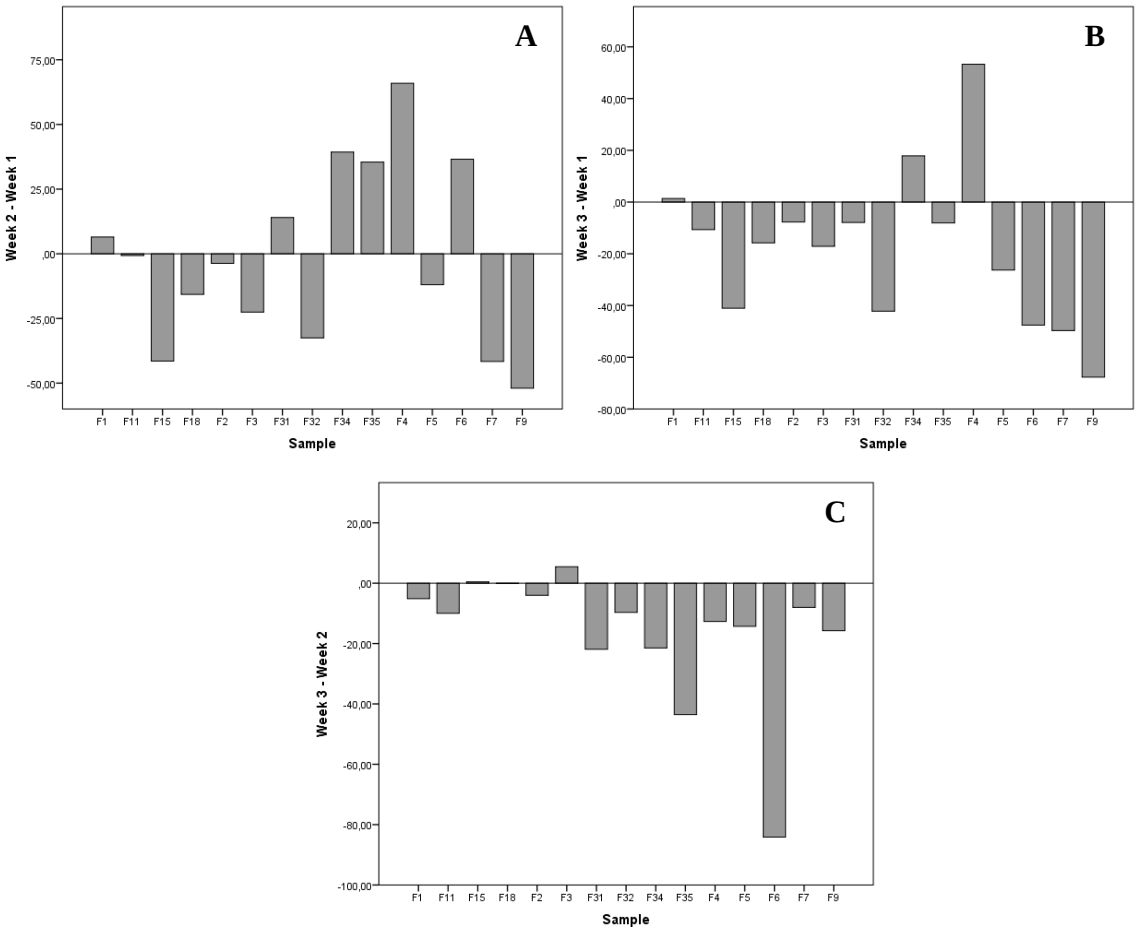

Figure 43 - **(A)** Bar plot sorted difference between TP2 and TP1 as a function of samples per total NAG concentration; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per total NAG concentration; and **(C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per total NAG concentration.

8.7.4. Antioxidant defense system

8.7.4.1. Total antioxidant status

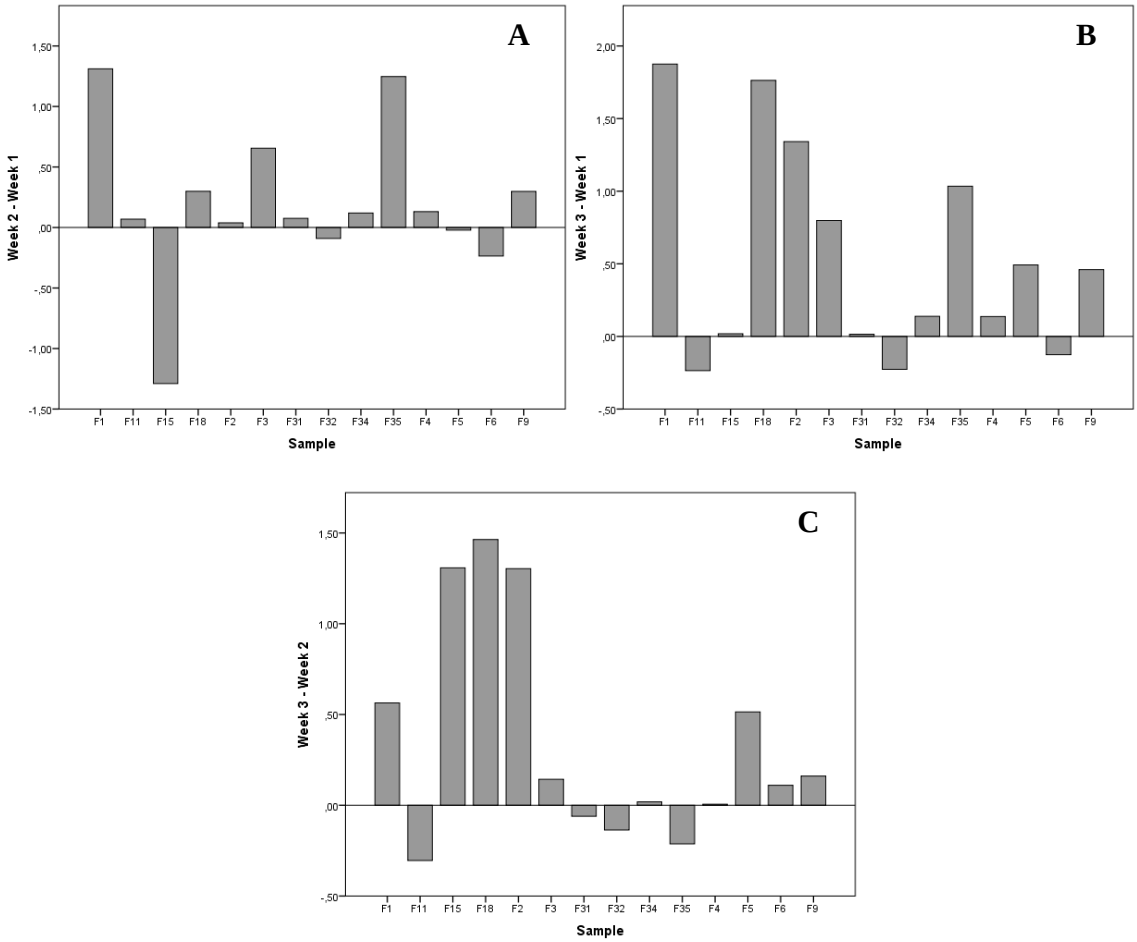

Figure 44 - **(A)** Bar plot sorted difference between TP2 and TP1 as a function of samples per TAS; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per TAS; and **(C)** Bar plot sorted difference between TP3 and TP2 as a function of samples per TAS.

8.7.4.2. SOD1 expression

Table 36 – Descriptive statistics of the SOD1 expression.

	SOD1 expression		
	TP1	**TP2**	**TP3**
N	14	14	14
Mean	2,61	1,42	1,40
Std. Deviation	4,11	1,16	1,46
Minimum	,66	,51	,12
Maximum	15,59	4,85	5,48

TP: time point; **SOD1**: superoxide dismutase 1

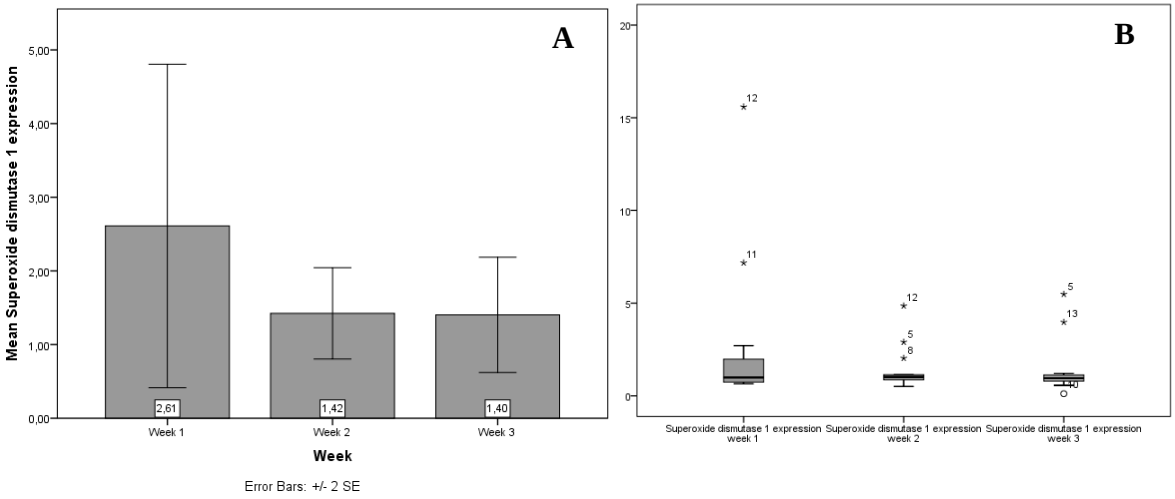

Figure 45 – **(A)** Bar graph and error bars of SOD1 expression; **(B)** Box plot and outliers of SOD1 expression the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. There is no significant differences between the three weeks (p=0,319).

8.7.4.3. GPx4 expression

Table 37 – Descriptive statistics of the GPx4 expression.

	GPx4 expression		
	TP1	**TP2**	**TP3**
N	14	14	14
Mean	,73	,65	,55
Std. Deviation	1,32	,75	,60
Minimum	,07	,04	,03
Maximum	5,26	2,90	2,25

TP: time point; **GPx4**: glutathione peroxidase 4

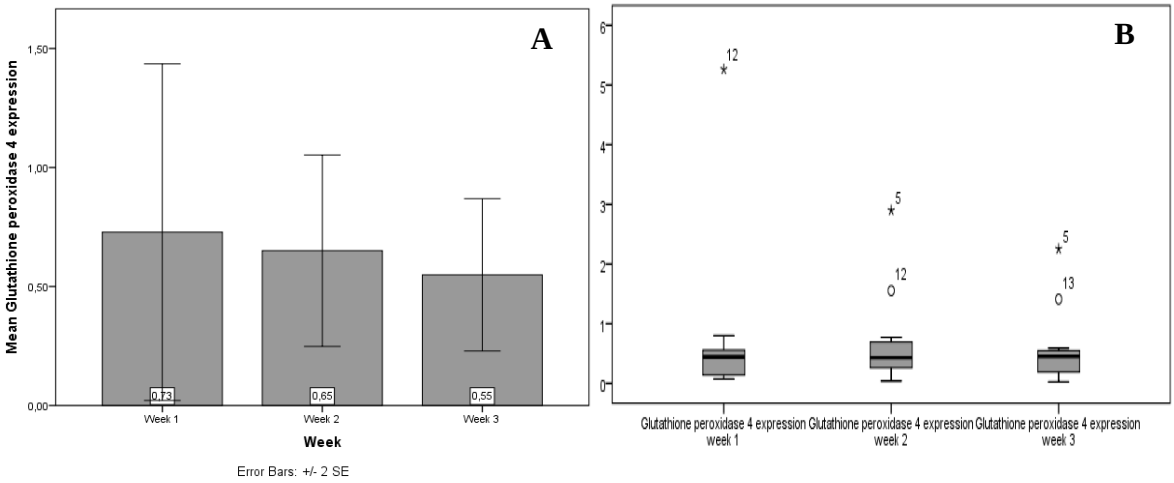

Figure 46 – **(A)** Bar graph and error bars of GPx4 expression; **(B)** Box plot and outliers of GPx4 expression the three weeks.

A Friedman test was used to determine if the differences between the three weeks were statistically significant. There is no statistical differences between the three weeks (p=0,424).

8.7.5. Protein oxidation

8.7.5.1. Carbonyl groups

Table 38 – Descriptive statistics of the carbonyl groups.

	Carbonyl groups		
	TP1	**TP2**	**TP3**
N	14	14	14
Mean	130,00	146,31	110,89
Std. Deviation	79,38	83,60	37,04
Minimum	56,27	76,69	68,01
Maximum	362,25	392,99	172,59

TP: time point

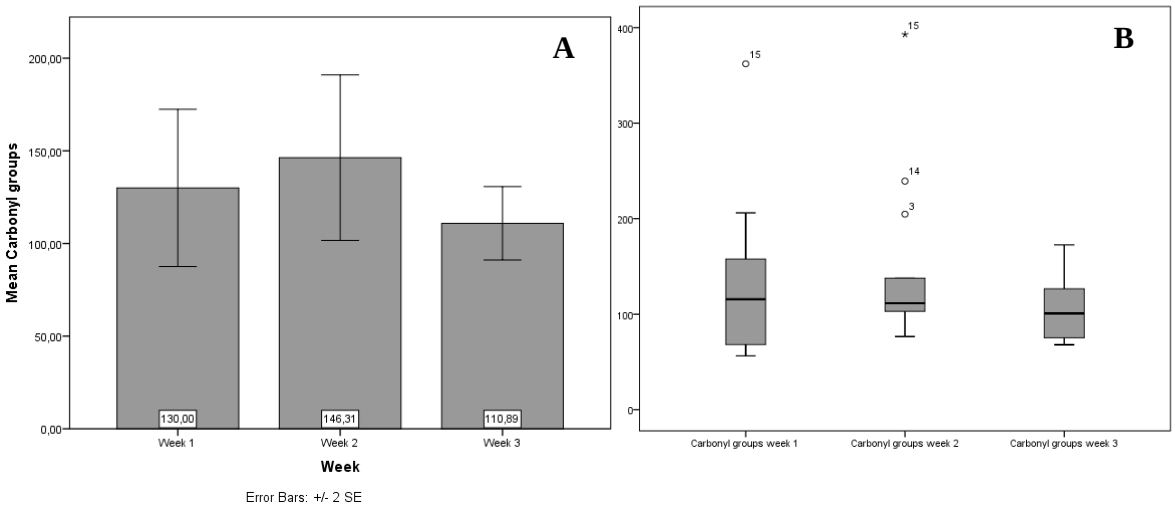

Figure 47 – **(A)** Bar graph and error bars of carbonyl groups; **(B)** Box plot and outliers of carbonyl groups at the three weeks.

A Friedman test was performed to determine if the differences between the three weeks were statistically significant. There is no significant difference between the three weeks (p=0,931).

8.7.5.2.3-Nitrotyrosine groups

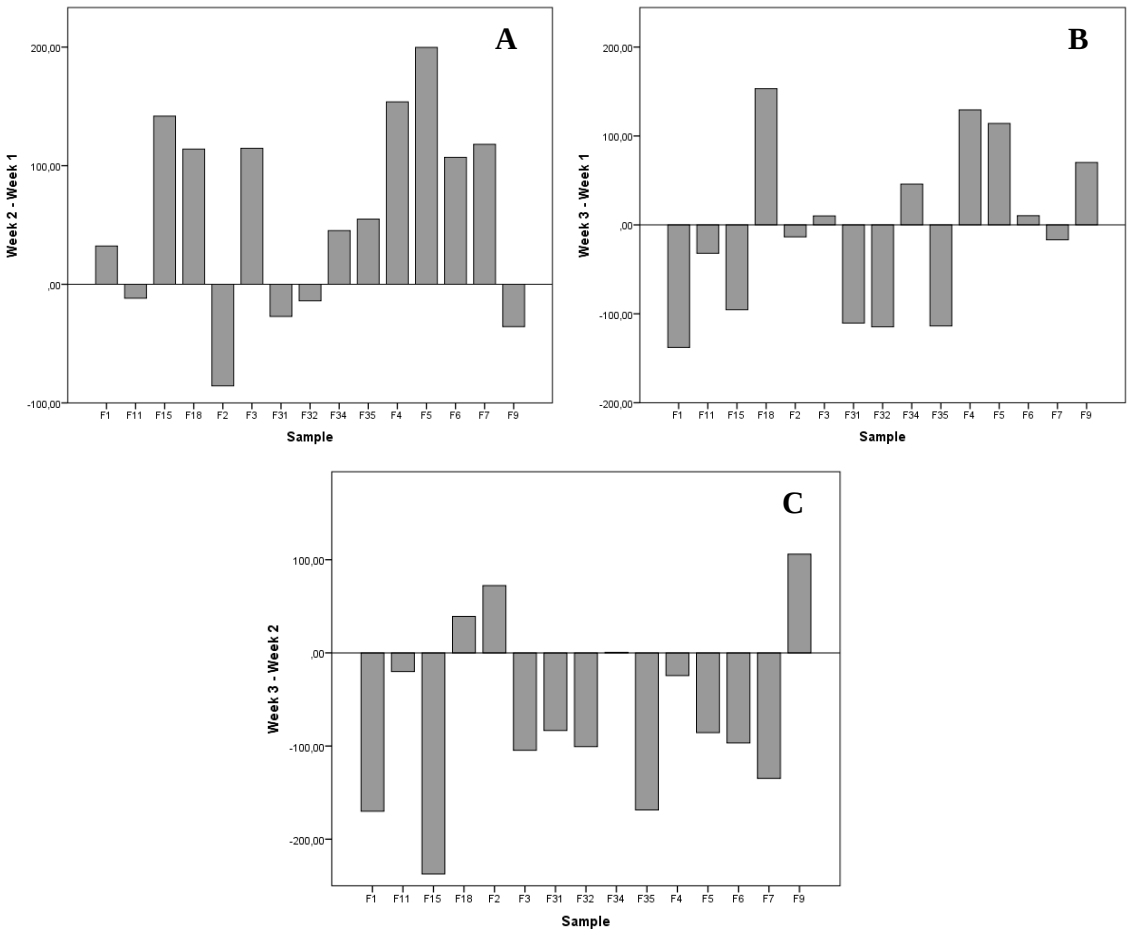

Figure 48 - **(A)** Bar plot sorted difference between TP2 and TP1 as a function of samples per 3-NT; **(B)** Bar plot sorted difference between TP3 and TP1 as a function of samples per 3-NT; and **(C)** Bar plot sorted difference between TP2 and TP3 as a function of samples per 3-NT.

8.7.6. Relation between seminal quality and stress oxidative stress parameters

8.7.6.1. Basic semen parameters and SO

Table 39 - Pearson correlation coefficient (r) with p-value.

		TAS	3-NT
Progressive motility	Pearson Correlation	-,239	-,205
	Sig. (2-tailed)	,132	,182
	N	41	44
Non progressive motility	Pearson Correlation	-,093	-,100
	Sig. (2-tailed)	,564	,519
	N	41	44
Imotility	Pearson Correlation	,189	,183
	Sig. (2-tailed)	,238	,234
	N	41	44
Normal sperm	Pearson Correlation	-,179	,016
	Sig. (2-tailed)	,283	,922
	N	38	41
Head defects	Pearson Correlation	,167	-,088
	Sig. (2-tailed)	,316	,586
	N	38	41
Tail defects	Pearson Correlation	,102	,280
	Sig. (2-tailed)	,543	,076
	N	38	41

TAS: total antioxidant status; **3-NT**: 3-nitrotyrosine

Table 40 - Spearman's Rank correlation coefficient (rs) with p-value.

		TAS	SOD1 expression	GPx4 expression	CG	3 -NT
Sexual abstinence	Correlation Coefficient	-,283	-,101	-,204	-,294	-,276
	Sig. (2-tailed)	,069	,525	,195	,059	,067
	N	42	42	42	42	45

Total number of spermatozoa/ejaculate	Correlation Coefficient	-,109	-,207	,154	,057	,062
	Sig. (2-tailed)	,493	,188	,330	,720	,685
	N	42	42	42	42	45

TAS: total antioxidant status; **SOD1**: superoxide dismutase 1; **GPx4**: glutathione peroxidase 4; **CG**: carbonyl groups; **3-NT**: 3-nitrotyrosine

Table 41 - Spearman's Rank correlation coefficient (r_s) with p-value.

		SOD1 expression	**GPx4 expression**	**CG**
Non progressive motility	Correlation Coefficient	-,060	-,016	-,175
	Sig. (2-tailed)	,709	,921	,273
	N	41	41	41
Normal sperm	Correlation Coefficient	-,008	,235	-,165
	Sig. (2-tailed)	,964	,156	,321
	N	38	38	38
Head defects	Correlation Coefficient	-,148	-,042	,242
	Sig. (2-tailed)	,376	,800	,143
	N	38	38	38
Midpiece defects	Correlation Coefficient	-,168	-,165	,174
	Sig. (2-tailed)	,312	,322	,295
	N	38	38	38
Tail defects	Correlation Coefficient	-,132	-,243	,171
	Sig. (2-tailed)	,430	,141	,304
	N	38	38	38

SOD1: superoxide dismutase 1; **GPx4**: glutathione peroxidase 4; **CG**: carbonyl groups

8.7.6.2. Basic semen parameters and accessory gland function

Table 42 - Pearson Correlation Coefficient (r) with p-value.

		[Citric acid]	Total [Fructose]
Progressive motility	Pearson Correlation	-,002	,138
	Sig. (2-tailed)	,989	,383
	N	43	42
Non progressive motility	Pearson Correlation	,151	,091
	Sig. (2-tailed)	,334	,567
	N	43	42
Imotility	Pearson Correlation	,019	-,183
	Sig. (2-tailed)	,904	,246
	N	43	42
Normal sperm	Pearson Correlation	-,033	,077
	Sig. (2-tailed)	,837	,639
	N	41	40
Midpiece defects	Pearson Correlation	,119	,044
	Sig. (2-tailed)	,457	,786
	N	41	40
Tail defects	Pearson Correlation	,236	-,122
	Sig. (2-tailed)	,138	,452
	N	41	40

Table 43 - Spearman's Rank correlation coefficient (r_s) with p-value.

		[Fructose]
Age	Correlation Coefficient	-,127
	Sig. (2-tailed)	,416
	N	43
Sexual abstinence	Correlation Coefficient	-,111
	Sig. (2-tailed)	,478
	N	43
Semen volume	Correlation Coefficient	-,216
	Sig. (2-tailed)	,165
	N	43
Sperm concentration	Correlation Coefficient	,237
	Sig. (2-tailed)	,125
	N	43

Total number of spermatozoa/ejaculate	Correlation Coefficient	,045
	Sig. (2-tailed)	,773
	N	43

Table 44 - Spearman's Rank correlation coefficient (r_s) with p-value.

		Total [Citric acid]	[Fructose]	[NAG]	Total [NAG]
Progressive motility	Correlation Coefficient	,259	-,056	,166	,281
	Sig. (2-tailed)	,090	,725	,294	,064
	N	44	42	42	44
Non progressive motility	Correlation Coefficient	,087	-,006	,063	,040
	Sig. (2-tailed)	,573	,972	,690	,794
	N	44	42	42	44
Imotility	Correlation Coefficient	-,246	,057	-,116	-,245
	Sig. (2-tailed)	,108	,721	,466	,108
	N	44	42	42	44
Normal sperm	Correlation Coefficient	-,217	,275	,322*	,075
	Sig. (2-tailed)	,174	,085	,043	,642
	N	41	40	40	41
Head defects	Correlation Coefficient	,054	-,117	-,209	,044
	Sig. (2-tailed)	,738	,473	,195	,784
	N	41	40	40	41
Midpiece defects	Correlation Coefficient	,096	-,024	-,284	-,122
	Sig. (2-tailed)	,551	,881	,075	,447
	N	41	40	40	41

Tail defects	Correlation Coefficient	-,197	,160	-,043	-,285
	Sig. (2-tailed)	,217	,324	,794	,071
	N	41	40	40	41

NAG: neutral α-glucosidase

8.7.6.3. Basic semen parameters and consumptions

Table 45 - Spearman's Rank correlation coefficient (r_s) with p-value.

		Alcohol consumption	**Tobacco consumption**
Sexual abstinence	Correlation Coefficient	-,019	-,098
	Sig. (2-tailed)	,903	,522
	N	45	45
Semen volume	Correlation Coefficient	-,011	,137
	Sig. (2-tailed)	,942	,371
	N	45	45
Sperm concentration	Correlation Coefficient	-,047	-,159
	Sig. (2-tailed)	,760	,296
	N	45	45
Total number of spermatozoa/ejaculate	Correlation Coefficient	-,070	-,052
	Sig. (2-tailed)	,650	,732
	N	45	45
Progressive motility	Correlation Coefficient	-,265	-,203
	Sig. (2-tailed)	,082	,186
	N	44	44
Non progressive motility	Correlation Coefficient	,120	-,011
	Sig. (2-tailed)	,440	,942
	N	44	44
Imotility	Correlation Coefficient	,228	,236
	Sig. (2-tailed)	,136	,122
	N	44	44
Normal sperm	Correlation Coefficient	-,205	-,163
	Sig. (2-tailed)	,199	,309
	N	41	41
Head defects	Correlation Coefficient	,054	,138
	Sig. (2-tailed)	,739	,390
	N	41	41

Midpiece defects	Correlation Coefficient	,239	,105
	Sig. (2-tailed)	,132	,512
	N	41	41
Tail defects	Correlation Coefficient	,278	,224
	Sig. (2-tailed)	,079	,159
	N	41	41

8.7.6.4. Oxidative stress parameters

Table 46 - Pearson Correlation coefficient (r) with p-value.

		TAS	**3-NT**
TAS	Pearson Correlation	1	,095
	Sig. (2-tailed)		,548
	N	42	42
3-NT	Pearson Correlation	,095	1
	Sig. (2-tailed)	,548	
	N	42	45

TAS: total antioxidant status; **3-NT**: 3-nitrotyrosine

8.7.6.5. Accessory gland function and oxidative stress parameters

Table 47 - Pearson Correlation coefficient (r) with p-value.

		[Citric acid]	**Total [Fructose]**
TAS	Pearson Correlation	-,051	-,202
	Sig. (2-tailed)	,753	,212
	N	41	40
3-NT	Pearson Correlation	,001	,029
	Sig. (2-tailed)	,995	,855
	N	44	43

TAS: total antioxidant status; **3-NT**: 3-nitrotyrosine

Table 48 - Spearman's Rank correlation coefficient (r_s) with p-value.

		[Citric acid]	Total [Citric acid]	[Fructose]	Total [Fructose]	[NAG]	Total [NAG]
SOD1 expression	Correlation Coefficient	-,026	,116	-,206	,025	,095	,191
	Sig. (2-tailed)	,872	,466	,203	,878	,558	,225
	N	41	42	40	40	40	42
CG	Correlation Coefficient	-,264	-,007	,105	,227	-,056	,063
	Sig. (2-tailed)	,095	,967	,521	,158	,732	,691
	N	41	42	40	40	40	42

SOD1: superoxide dismutase 1; **CG**: carbonyl groups; **NAG**: neutral α-glucosidase

Table 49 - Spearman's Rank correlation coefficient (r_s) with p-value.

		Total [Citric acid]	Total [NAG]
TAS	Correlation Coefficient	-,078	-,266
	Sig. (2-tailed)	,625	,089
	N	42	42
3-NT	Correlation Coefficient	,009	-,174
	Sig. (2-tailed)	,952	,254
	N	45	45

TAS: total antioxidant status; **3-NT**: 3-nitrotyrosine; **NAG**: neutral α-glucosidase

8.7.6.6. Consumptions and oxidative stress parameters

Table 50 - Spearman's Rank correlation coefficient (r_s) with p-value.

		Alcohol consumption	Nicotine consumption
TAS	Correlation Coefficient	,055	-,238
	Sig. (2-tailed)	,727	,130
	N	42	42
SOD1 expression	Correlation Coefficient	-,078	-,019
	Sig. (2-tailed)	,625	,906
	N	42	42
GPx4 expression	Correlation Coefficient	-,154	-,016
	Sig. (2-tailed)	,330	,920
	N	42	42
CG	Correlation Coefficient	,137	,194
	Sig. (2-tailed)	,387	,218
	N	42	42

TAS: total antioxidant status; **SOD1**: superoxide dismutase 1; **GPx4**: glutathione peroxidase 4; **CG**: carbonyl groups.

8.7.6.7. Consumption and Accessory gland function

Table 51 - Spearman's Rank correlation coefficient (r_s) with p-value.

		Alcohol consumption	Nicotine consumption
[Citric acid]	Correlation Coefficient	,007	-,106
	Sig. (2-tailed)	,966	,493
	N	44	44
Total [Citric acid]	Correlation Coefficient	-,065	,110
	Sig. (2-tailed)	,673	,473
	N	45	45
[Fructose]	Correlation Coefficient	-,392**	,059
	Sig. (2-tailed)	,009	,706
	N	43	43
Total [Fructose]	Correlation Coefficient	-,339*	,186
	Sig. (2-tailed)	,026	,232
	N	43	43

[NAG]	Correlation Coefficient	-,144	,124
	Sig. (2-tailed)	,355	,429
	N	43	43
Total [NAG]	Correlation Coefficient	-,151	,249
	Sig. (2-tailed)	,321	,099
	N	45	45

NAG: neutral α-glucosidase